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Assessment of plasma stability of novel anti-Alzheimer's agents

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Zusammenfassung

Donepezil und Rivastigmin sind zwei bedeutsame cholinerge Therapeutika, die primär zur Linderung der kognitiven Symptome bei Alzheimer und anderen Demenzformen zugelassen sind. Die Notwendigkeit neuer Alzheimer-Medikamente ergibt sich aus der begrenzten Halbwertszeit und den auftretenden Nebenwirkungen. Für diese Studie wurden 26 neue und vielversprechende Donepezil und Rivastigmin Hybride auf deren *in vitro* Plasmastabilität in Human- und Rattenplasma untersucht. Dabei wurden die Plasmaproben über einen Zeitraum von 24.00 h bei einer Temperatur von 37 °C inkubiert und mit UHPLC oder LC-HRMS vermessen. Wie erwartet, zeigten die meisten Analyten, sowie AN13 (89.5±1.4% in Humanplasma and 75.3±9.4% in Rattenplasma), einen stärkeren Abbau in Rattenplasma als in Humanplasma nach 24.00 h Inkubation. Die Derivate GIA25, ROS111 und VIT35 zeigten ein gegenteiliges Verhalten. Nach 24.00 h Inkubation verblieben 67.9±6.5% von GIA25 in Humanplasma, verglichen mit den 74.7±4.1% in Rattenplasma. Ähnlich blieben 84.3±2.6% von ROS111 und 67.9±6.5% von VIT35 im Humanplasma, während im Rattenplasma 89.6±3.4% und 74.7±4.1% erhalten geblieben sind. Der Analyt ROS131 zeigte die kürzeste berechnete *in vitro* $t_{1/2}$, mit über 24.00 h im Humanplasma (circa 39 h) und 17 h im Rattenplasma. Grundsätzlich haben die Derivate eine größere Plasmastabilität als der Parentcompound Rivastigmine ($t_{1/2}$ = 1 h) gezeigt. Zwanzig Analyten übertrafen mit ihrer berechneten $t_{1/2}$ Humanplasma *in vitro* die *in vivo* $t_{1/2}$ von Donepezil ($t_{1/2}$ = 80 h) im Menschen, darunter AN13 ($t_{1/2}$ = 173 h), AN15 ($t_{1/2}$ = 161 h) und ROS151 ($t_{1/2}$ = 169 h). Die Analyten haben in dieser Stabilitätsassay den Parentcompound Rivastigmin durchwegs übertroffen, in Hinblick auf die $t_{1/2}$. In den meisten Fällen wiesen die Derivate eine höhere $t_{1/2}$ im Humanplasma als der Parentcompound Donepezil.

Abstract

Donepezil and rivastigmine are two cholinergic therapeutics that are approved for the treatment of cognitive symptoms associated with Alzheimer's disease and other forms of dementia. The need for optimised Alzheimer's drugs arises from the limited half-life and the adverse effects that occur. In this study, novel and promising 26 donepezil and rivastigmine hybrids were analysed for their *in vitro* plasma stability in human and rat plasma. The plasma samples were incubated at a temperature of 37 °C for a period of 24.00 h and measured using UHPLC or LC-HRMS. As expected, most of the analytes including AN13 (89.5±1.4% in human plasma and 75.3±9.4% in rat plasma) degraded faster in rat plasma than in human plasma following the 24.00 h of incubation. The derivatives GIA25, ROS111 and VIT35 showed the opposite trend. After 24.00 h incubation 67.9±6.5% of GIA25 remained in human plasma compared to 74.7±4.1% in rat plasma. Similarly, 84.3±2.6% of ROS111 and 67.9±6.5% of VIT35 remained in human plasma, compared to 89.6±3.4% and 74.7±4.1%, respectively, in rat plasma. The analyte ROS131 had the shortest calculated *in vitro* $t_{1/2}$ with more than 24.00 h in human (approximately 39 h) and 17 h in rat plasma. Overall, the derivatives demonstrated higher plasma stability than the parent compound rivastigmine ($t_{1/2}$ = 1 h) . Twenty analytes surpassed the human *in vivo* $t_{1/2}$ of donepezil ($t_{1/2}$ = 80 h) with their calculated $t_{1/2}$ in the human plasma *in vitro* study including AN13 ($t_{1/2}$ = 173 h), AN15 ($t_{1/2}$ = 161 h) and ROS151 ($t_{1/2}$ = 169 h). The compounds consistently outperformed the parent compound rivastigmine in this stability assay in terms of $t_{1/2}$. In most cases, the derivatives displayed a superior $t_{1/2}$ in human plasma than the parent compound donepezil.

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1. Introduction

1.1. Donepezil hydrochloride

Donepezil hydrochloride (DOH), (*RS*)-2-[(1-benzyl-4-piperidyl)methyl]-5,6-dimethoxy-2,3-dihydroinden-1-one, E2020, (Figure 1.), is a racemic substance which was introduced 1997 by the EMA for the treatment of Alzheimer's disease [1]. Alzheimer's disease is a progressive neurological disorder primarily associated with older adults [2].

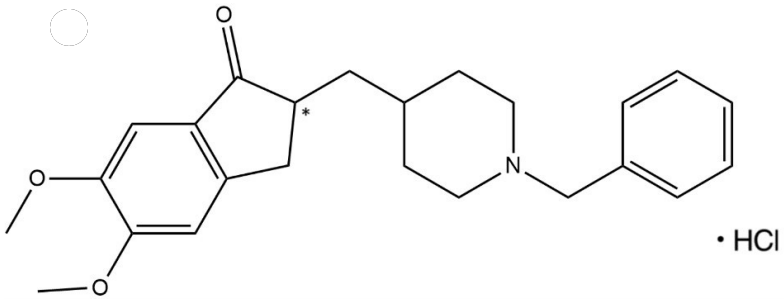


Figure 1. Structure of DOH. * Denotes the chiral centre.

The research for new acetylcholinesterase inhibitors (AChE inhibitors) began because of the limitations of rivastigmine due to poor oral availability and the hepatotoxic effects of tacrine. Using a *N*-benzylpiperazine derivative over 700 derivatives were synthesized. These led to the discovery that replacing the *N*-benzylpiperazine with a *N*-benzylpiperidine moiety increased the AChE inhibitation. After further modifications, the new compound E2020 was discovered, which is now known as DOH. This compound has found to be an AChE inhibitor (IC_{50} = 5.7 nM) and an butyrylcholinesterase inhibitor (BuChE, IC_{50} = 7138 nM), with an higher affinity for BuChE. DOH is more selective than tacrine (IC_{50} AChE / IC_{50} BuChE = 0.9) and physostigmine (IC_{50} AChE/IC_{50} BuChE = 11.9). Responsible for the selective inhibition of AChE seems to be the *N*-benzylpiperidine and indanone moieties [3].

The maximum plasma concentration c_{max} of DOH is reached approximately 3-4 h after oral administration [4]. The long *in vivo* half-life ($t^1/_2$) of DOH (80 h) in humans results in a slow approach to steady state. When administering one dose a day steady state is reached between 2 to 3 weeks after initiation of dose administration. This leads to a predictable stable pharmacokinetic profile during repeated regular intake, resulting in simplification of the use. [5] DOH is a racemate (*R*-DOH, *S*-DOH) on account of the asymmetric carbon atom (see Figure 1). The enantiomers have varying extents of AChE inhibition *in vivo* and *in vitro*. The plasma levels of *R*-DOH are lower than those of S-DOH in human plasma. The higher plasma concentration of *S*-DOH result in a better AChE inhibition, compared to the *R*-DOH enantiomer [6]. In rat plasma the *in vivo* $t^1/_2$ is 3 h after i.v. administration [7].

To this date, there are more than ninety countries with approval of DOH usage for Alzheimer's disease. In Austria two preparations of the racemic DOH (5 mg/day and 10 mg/day) are available and one additional (23 mg/day) in the USA [8].

DOH undergoes extensive first-pass metabolism in the liver, primarily mediated by CYP3A4 and to a lesser extent by CYP2D6. The metabolites formed by O-dealkylation, hydroxylation, *N*-oxidation, hydrolysis and O-glucuronidisation have nearly the same $t^1/_2$ as the parent drug in human plasma. Two out of the four major DOH metabolites found in human plasma are known to be active, whereas there are some which have not been identified yet. The active metabolite 6-*O*-desmethyl DOH, see Figure 2., was identified by radiolabelling, which shows similar potency in inhibition of AchE as its parent compound [9]. The primary route of excretion for DOH and its metabolites takes place via the urine [10].

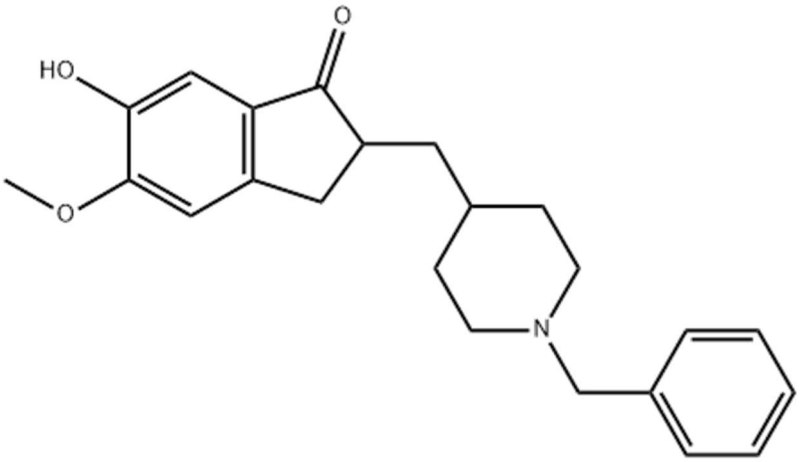


Figure 2. The active metabolite 6-O-desmethyl DOH formed through dealkylation by CYP2D6.

Medications with 5 mg/day and 10 mg/day DOH were approved for moderate and severe Alzheimer's disease in 1997 by the EMA [8]. The FDA approved 23 mg/day DOH with sustained release formulation resulting in a slower rise to c_{max}. This change in formulation helps to reduce adverse effects [11]. The benefits of the patients on 23 mg/day were no greater than on 10 mg/day, and benefits on the 10 mg/day dose were marginally larger than on the 5 mg/day dose [8].

In Austria DOH is approved for Alzheimer's disease with 5 mg/day at the beginning of the treatment with the possibility to increase the dose up to 10 mg/day. The administration must be monitored by a doctor and the daily intake should be monitored by a caregiver. Furthermore, the clinical benefit of DOH should be regularly assessed because the treatment should only continue if the patient derives therapeutic benefit from it [4].

Patients with renal dysfunction do not need changes in the dosage as the clearance of DOH is not affected. However, due to the potential for increased drug levels in patients with mild to moderate hepatic impairment the dose should be chosen based on individual tolerability [10].

The most common adverse events effecting one in ten patients include diarrhoea, fatigue, nausea, vomiting, and insomnia. Serious side effects like abnormal heart rhythms, urinary incontinence and seizures were documented in at least one out of 100 patients. DOH is contradicted in patients allergic to DOH and piperidine derivatives. A physician should be consulted regarding the intake of DOH if the patient has been diagnosed with ulcers, seizures, heart diseases, asthma or other long-term lung diseases, hepatitis, or pregnancy [4].

DOH is not used solely for Alzheimer's disease but is also administered as an off-label drug. Several studies have shown the positive effect of DOH in the treatment of cognitive and

behavioural symptoms in people with Lewy body dementia. New research suggests that DOH therapy may improve memory impairment in patients with traumatic brain injury. Studies indicate that DOH may improve cognitive abilities in patients with vascular dementia, although its effect on overall functioning appears to be limited. Furthermore, there is evidence that DOH may improve cognition, executive function, and general health in people with dementia associated with Parkinson's disease [12].

1.2. Rivastigmine

Rivastigmine, (*S*)-{3-[α-(dimethylamino)ethyl]phenyl}-*N*-ethyl-*N*-methylcarbamat, (Figure 3.) was introduced 1998 in Europe for the treatment of mild to moderate Alzheimer's disease, Parkinson's disease and patients with dementia [13]. Rivastigmine is available in more than 80 countries either as a capsule or as a patch [14].

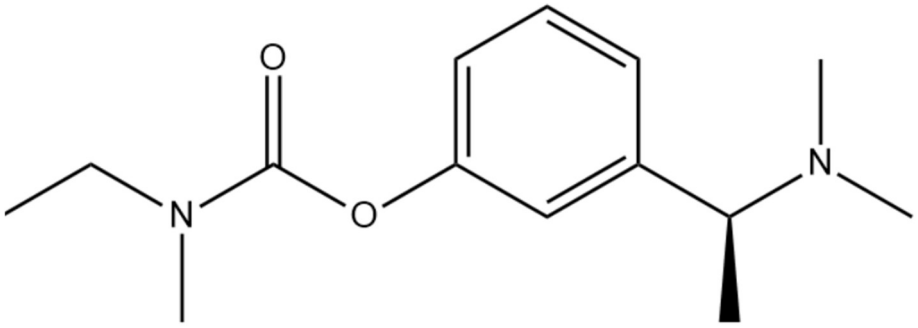


Figure 3. The structure of *S*-rivastigmine.

In the early 1930s it was discovered that physostigmine and related carbamates inhibit AChE strongly. During the research for simplified physostigmine derivatives, four phenylcarbamates were synthesised. Miotine (Figure 4.) is the meta derivative, that was proven to be the most active compound. Despite miotine's potency, the strong adverse health effects as e.g. bradycardia and respiratory arrest, soon put a hold to further research the compound [15].

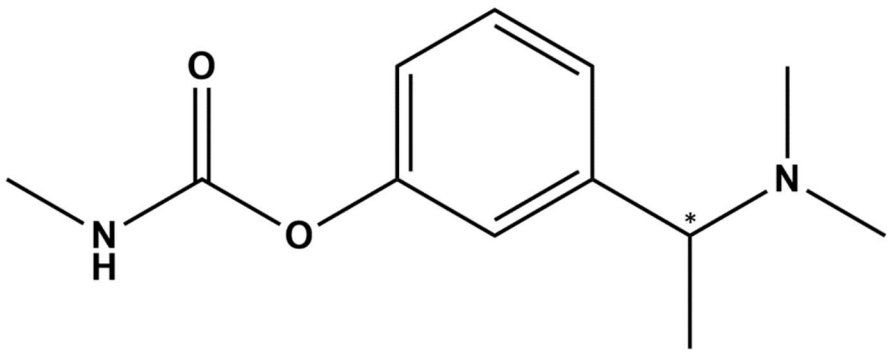


Figure 4. The structure of miotine. The * denotes the chiral centre.

In the early 1980s, Dr. Weinstock reevaluated the carbamate structures in search for a drug for treating the Alzheimer's disease. There was a modification made to miotine's carbomyl *N*-substituent and a methylethyl group was added, resulting in the discovery of the racemate rivastigmine [16]. Several carbamate derivatives were evaluated and the rivastigmine racemate demonstrated satisfactory *in vivo* activities. The more active *S*-enantiomer was developed into the Alzheimer's disease drug rivastigmine [15].

Rivastigmine is solely used for symptomatic treatment of Alzheimer's disease, it only serves to mitigate the symptoms. But cognitive improvement can be expected due to an increase of nicotinic receptor expression, as well as a change of the ratio of AChE isoforms in the central nervous system (CNS) [14].
Rivastigmine, a semi-synthetic derivative of physostigmine, is a dual inhibitor of AChE and BuChE, which accounts for 10% of the total cholinesterase in the human brain [17] . Preclinical and clinical studies have found that rivastigmine induces greater inhibition of AChE in the CNS than in the periphery. Furthermore, rivastigmine inhibits AChE more potent in the cortex and hippocampus, where the brain is most affected by Alzheimer's disease [18]. Rivastigmine mimics acetylcholine and binds both anionic and esteratic sites of AchE. It does not dissociate after hydrolysis. The hydrolysed rivastigmine leaves the esteratic site of AChE carbamylated for an extended period of time, resulting in prolonged enzyme inhibition. Due to structural properties rivastigmine displays a greater potency for the globular form G1 of AChE. The isoform G1 is expressed more often in brains of patients with Alzheimer's disease, while the G4 tetramer is more abundant in healthy young individuals [14].
After a single oral dose, the enzyme inhibition lasts for up to 12 h exhibiting equal potency to AChE and BuChE [19]. The fast and almost complete absorption without food intake results in a t_{max} of 0.8 – 1.2 h and is increased with food intake to t_{max} 1.4 – 1.6 h. Thereby increasing the bioavailability by 30% and reducing the c_{max} about 30%, resulting in fewer adverse effects on the gastrointestinal tract [14].

In animals and humans rivastigmine is metabolized rapidly and extensively, with a $t_{1/2}$ in rats under 1 h. The plasma elimination $t_{1/2}$ of humans is 1 h. The hydrolysation to the active decarbamylated metabolite NAP 226-90 (Figure *5.*) is mediated by cholinesterase. NAP 226-90 reaches c_{max} two h after administration and can undergo *N*-demethylation, sulfate conjugation, or both. The major route of elimination is renal. In humans, the sulfate conjugate was the principal metabolite found in urine, where the parent compound cannot be detected [18].

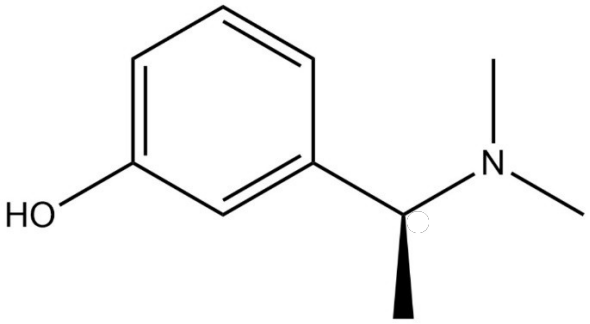


Figure 5. The hydrolysed metabolite of rivastigmine, NAP 226-90.

1.5 mg, 3.0 mg, 4.5 mg and 6.0 mg rivastigmine capsules are available in the European Union and are administered two times a day, with breakfast and dinner. In the first few weeks dose titration is performed until the required final dose is reached. Each phase of adaptation lasts about two weeks. This reduces adverse effects such as nausea, vomiting and diarrhoea [19]. The capsule is administered bidaily to maximise benefits with minimal side effects in the dose range of 3 – 6 mg [14]. The most common side effects occurring in more than one out of ten people include vertigo, gastrointestinal problems, tremor and loss of appetite [20].

To overcome limitations of oral administration and compliance the FDA approved a transdermal patch of rivastigmine with a dose of 13.3 mg for the once-daily treatment of moderate to severe Alzheimer's disease in 2013 [21]. Today 4.6 mg/24 h, 9.5 mg/24 h and 13.3 mg/24 h transdermal patches are available in Europe. The initial dose of 4.6 mg/24 h is administered over 4 weeks and increased to the recommended daily dose of 9.5 mg/24 h. If the patient

shows a substantial cognitive deterioration the dose can be increased by a physician to 13.3 mg/24 h [22].
The chemical composition of rivastigmine is ideal for transdermal applications due to its small molecular size and hydrophilic and lipophilic properties. This results in good barrier penetration, allowing rivastigmine to access the bloodstream and cross the blood-brain barrier [14]. The absorption of rivastigmine from the transdermal patch is slow. After the first administered dose, detectable plasma levels can be observed after 0.5 – 1 h. The c_{max} is reached after 10 – 16 h and the plasma level decreases slowly over the remaining 24-hour interval. When steady state is reached, the minimum plasma concentration is about 50% of c_{max}. In contrast, with oral administration the plasma concentration reaches almost zero between the morning and evening dose [22]. Transdermal administration lowers the c_{max} and prolongs the t_{max} resulting in reduced fluctuation of plasma concentration compared to oral administration [23]. The achieved steady plasma level results in higher rates of medication adherence, good safety, and tolerability. One compliance issue can occur when not removing the old patch during application of the new one and toxic rivastigmine plasma levels can be reached. Caregivers need to be informed of this potential risk in order to be able to act accordingly [14]. Signs of toxicity include miosis, vertigo and increased muscle weakness. The most common adverse effects of the transdermal patch are skin reactions at the applicated area and gastrointestinal problems, occurring in more than one out of one hundred patients [22].

1.3. DOH and rivastigmine analogues

In process of finding a new lead structure several molecules were synthesised by the team of Leonardo Brunetti and Rosalba Leuci from the University of Bari during their PhD. These derivatives were designed as molecular hybrids of DOH and rivastigmine. This study focuses on comparing the plasma stabilities of the 26 derivatives in human and rat plasma, whereas DOH itself was used as internal standard (IS) in the measurements.

1.4. Plasma stability of drugs

Plasma stability is essential for the success in medical treatment of new compounds. Issues such as fast plasma degradation and low target activity often require improvement. The actions of the plasma metabolization influence the $t^1/_2$, pharmacokinetic profile and the concentration of the drug at the target. Most drugs are metabolised by members of the CYP450 family and esterases. Drugs with esters, amides, carbamates, lactones, lactams, sulfates, sulfonamides, phosphates, peptides and peptide mimetics are more susceptible for hydrolysation by plasma enzymes. The rate of degradation is dependent on the functional group - esters are hydrolysed faster than amides and sulfonamides, which in turn are hydrolysed faster than carbamates [24]. In order to overcome plasma stability problems *in vivo* it is a straightforward approach to exchange more sensitive targets against less susceptible groups [25].

The proteins and lipids contained in biological matrices show additional opportunities for non-covalent binding [26]. The transport of drugs to its target is hindered by excessive plasma protein binding and shows significant influence on the plasma stability. In addition, the protein bound molecules cannot undergo glomerular filtration [27].

Plasma contains various proteins with albumin being the most abundant, followed by *α*-1-acid glycoprotein and to a lesser degree, globulins and lipoproteins. Albumin binds with high affinity acidic and neutral drugs [28].
DOH is 96% protein-bound, with approximately 75% binding to albumin and approximately 21% binding to *α* -1-glycoprotein. The volume of distribution of DOH is 11.8 ± 1.7 L/kg for a 5-mg dose and 11.6 ± 1.91 L/kg for a 10-mg dose which suggests extensive distribution in extravascular compartments. With both doses the mean cerebral spinal fluid concentration reaches 15.7% [9].

Rivastigmine displays low protein binding with 40%, about 40% to red blood cells. The volume of distribution of rivastigmine lies between 1.8 and 2.7 L/kg, suggesting a wide distribution throughout the body. The main metabolite, NAP 226-90, has a volume of distribution of 4.3 - 5.9 L/kg [19].

The occurrence of enzyme and their activity in plasma can vary due to differences depending on species, gender and even ethnicity in humans. It was shown by Hale *et al.* (2000) that the highest hydraulic rates occur in humans followed by dogs and rats [29].
Differences in metabolism of DOH were noted by Noetzli *et al*. (2010) with an 18% reduced plasma clearance in elderly female patients compared with the male patients, which results in higher plasma $t^1/_2$ in females than in males. Whereas they could confirm that age, renal function, and nicotine consumption had no significant influence on DOH clearance [30].
The elimination $t^1/_2$ of rivastigmine is slightly prolonged in elderly (0.88 – 1.25 h) compared to young adults (0.80 – 0.99 h). For patients with renal or hepatic impairment there are no specific dose recommendations because the dose will be titrated to the individuals maximum tolerated dose [18].

The ethnicity of the patients was not found to impact the metabolization of DOH. 37.9% of the Chinese population carry a CYP2D6*1 variant, which increases the activity of the enzyme and 51.3% have the CYP2D6*10 variant, which decreases CYP2D6 activity. On the contrary, 98% of poor metabolizing Caucasians have CYP2D6*3, CYP2D6*4, and CYP2D6*5 variants, which are rarely found in Chinese patients.
A study found that patients with the CYP2D6*1/10 or CYP2D6*10/10 genotype have a better cognitive response and a higher plasmatic concentration of DOH after six months compared to subjects with CYP2D6*1/1. In addition, patients with the CYP2D6*10 allele have fewer adverse effects [31].
APOE ε4 allele is a susceptibility gene for Alzheimer's disease, but the effects depend on differences in sex, age, and ethnicity. Taiwanese patients with the APOE ε4 allele treated with rivastigmine displayed worse cognition, compared to Caucasians. Furthermore, Taiwanese people show a lower prevalence of APOE ε4 allele compared to other ethnicities [32]. There is evidence to suggest a potential influence of sex on treatment outcomes. Women appear to exhibit greater sensitivity to therapy and experience less cognitive decline than men. ESR1 may also be a contributing factor to the interindividual variability in response to treatment with DOH and rivastigmine [33].

Before *in vivo* studies can be conducted with newfound compounds, they need to undergo *in vitro* testing. Frequently the samples of *in vitro* plasma stability essays do not get measured right away. The enzymatic activity of the plasma must be inhibited to prevent the further degradation of the substance [34]. One way to achieve this is protein precipitation. Protein precipitation involves adding an organic solvent, such as methanol (MeOH), acetonitrile (ACN) or dimethyl sulfoxide (DMSO) to high protein matrices. The precipitation occurs due to the change of interactions between protein and the aqueous environment. The proteins denature, aggregate, and precipitate out of solution. The remaining protein particles can be removed from the sample by filtration or centrifugation. This method provides a simple and fast sample cleanup [35].

1.5. Alzheimer's disease

Alzheimer's disease is by far the most common cause of dementia worldwide. The two most common pathophysiological changes are β-amyloid-containing plagues and tau-containing neurofibrillary tangles [36]. These induce negative loss of synaptic homeostasis, neuronal network integrity and lead to synaptic and neuronal loss, which worsens cognitive functions [37]. The progressive cognitive decline is indicated by memory loss, impairments in attention, concentration, and judgment. In addition, patients have significantly reduced communication and physical abilities [11]. The quality of life decreases with the degree of cognitive impairment,

ranging from giving up hobbies to not being able to sustain one's own life, often resulting in a family member being the primary caregiver. In this way Alzheimer's disease takes a mental toll on the whole family [2].

Non modifiable risk factors for Alzheimer's disease are age and genetics. The identified genes are dominantly inherited mutations in the amyloid precursor protein, presenilin 1 and 2PSEN2. With the dominantly inherited genes the onset is 40 years earlier, but when they are inherited with mutations the onset always occurs aged 65 or younger [37].
Modifiable risk factors for Alzheimer's disease in early life include obesity, low HDL cholesterol, brain injury and alcohol abuse. In later life smoking, social isolation and low physical activity increase the risk of Alzheimer's disease. There are no scientifically proven pharmacological or non-pharmacological ways to prevent Alzheimer's disease, but lifestyle changes and mental exercise may improve or delay cognitive impairment [36].
The current pharmacological approaches to the treatment are the three AChE inhibitors rivastigmine, galantamine, and DOH [11]. AChE is primarily used in the autonomic nervous system as a neurotransmitter for exocytosis. The inhibition of AChE increases the concentration of acetylcholine in the neuromuscular junctions and in the parasympathetic nervous system. This leads to higher ACh binding to nicotinic and muscarinic receptors and thereby enhancing cholinergic neurotransmission [38].

The research in future treatments involves targeting the pathologies. Hyperphosphorylation of tau seems to be one of the direct causes of symptoms of Alzheimer's disease. The safety and efficacy of different tau vaccines in animal models has already been proven. A small study testing an anti-tau drug has shown a positive immune response in humans. Different early phase trials of drugs targeting tau are currently conducted [36].
Preventing the development of amyloid plaque poses a different research approach to the pathology of Alzheimer's disease [37].

It is important to push the research against Alzheimer's disease further, to find a drug that can cure the disease. The goal in research is to strive for better efficiency, fewer adverse effects, and drug-drug interactions and better patient compliance.

1.6. RP-UHPLC

High-performance liquid chromatography (HPLC) is an analytical method used to separate, identify, and quantify compounds in mixtures. In general, a HPLC unit consists of a solvent reservoir, solvent degasser, high pressure pumps, autosampler, column oven, and a detector (i.e. mass spectrometry (MS), Ultraviolet–visible spectrophotometry (UV-Vis)). The collected data is transformed by software to a chromatogram. Every sample elutes in its specific time (retention time, RT) and has an area proportional to its amount [39].

In reversed phase HPLC (RP-HPLC) the mobile phase is moderately polar, and the stationary phase is non-polar. The more hydrophobic the compounds are, the more they get retained by the stationary phase. Ultra-high performance liquid chromatography (UHPLC) is used to further optimise separation by implementing shorter columns with smaller particle size, resulting in the necessity of a higher pump pressure [40].

The addition of salts or acids (e.g., formic acid) to mobile phase changes the pH and thus the ionization of the compounds, often resulting in better separation and resolution [39].

1.7. Mass spectrometry

In mass spectrometry (MS), molecules are transformed into positive or negative ions, in order to measure the mass-to-charge ratio. The charged molecules or fragments are separated according to their masses and relative intensities which are registered in a mass spectrum [39].

1.7.1. MS – ion source

It is important for the mass spectrometer to provide a high ionization efficiency and a high stability of ions for the mass analysers. The ionisation source should be able to control the degree of fragmentation by varying the internal energy deposited on ions during ionization. Often the ion source is paired with chromatography methods, most often the UHPLC [41].

In this study electrospray ionization (ESI) was used as ion source. An electrospray is the dispersion of a liquid into very small droplets in an electric field. The analyte solution is sprayed from a capillary (50 to 100 µm) at atmospheric pressure into an electric field created by applying a high voltage (3 to 6 kV) between the capillary and the counter electrode, forming small droplets. Solvent molecules are evaporated by preheated N_2 causing the charge density to increase. Once the stability limit (Rayleigh limit) is exceeded, the repulsive forces of identical charges lead to an explosive decay (Coulomb explosion) and the droplets fission into even smaller ones in the range of a few nm. Additional desolvation leads to the release of ions into the gas phase. This method is characterised by its soft ionisation method resulting in mostly molecular ions with minimal fragmentation [40].

For basic analytes the positive ion mode can be used. Besides H^+ ions, adducts like Na^+, K^+ or NH_4^+ are formed resulting in forms of protonated molecular ions $[M+H]^+$. Acidic analytes usually require the negative ion mode, forming either deprotonated molecular ions $[M-H]^-$ or adducts with $CHOO^-$, CH_3COO^- or Cl^-. This study was conducted using solely the positive ion mode [39].

1.7.2. MS – mass analyser

The mass analyser separates the ions depending on their mass-to-charge ratio. There are different types of mass analysers, like quadrupole, ion trap, time-of-flight (TOF), and Fourier transform analysers. There is no mass analyzer suitable for all applications, therefore the analysers are often used in tandem. In this study quadrupole - quadrupole - time-of-flight (qq-TOF) was used [41].

The quadrupole consists of four cylindrical or hyperbolic rods parallel to each other. The rods in opposition to each other are electrically connected and a radio frequency (RF) is applied. A direct current (DC) potential is layered over the RF potential. Depending on the DC/RF ratio only ions with a particular m/z will have stable trajectories. Ions with unstable trajectories collide with the rods and are filtered out [40].

In a TOF mass spectrometer the ions generated are accelerated through a known potential and travel through a flight tube in order to reach the detector. The m/z values are directly related to the travel time of the ions. Heavier ions take longer to reach the detector, while light ions reach the detector ahead of them. Modern TOF analysers compensate for different the initial kinetic energy of the ions with a reflectron field. Ions with higher kinetic energy penetrate the reflectron field deeper, thus resulting in a longer flight time back to the detector than less energetic ions. The advantages of TOF include a high mass range, high resolution, good mass accuracy and the almost simultaneous detection of ions [39].

1.8. Research aim

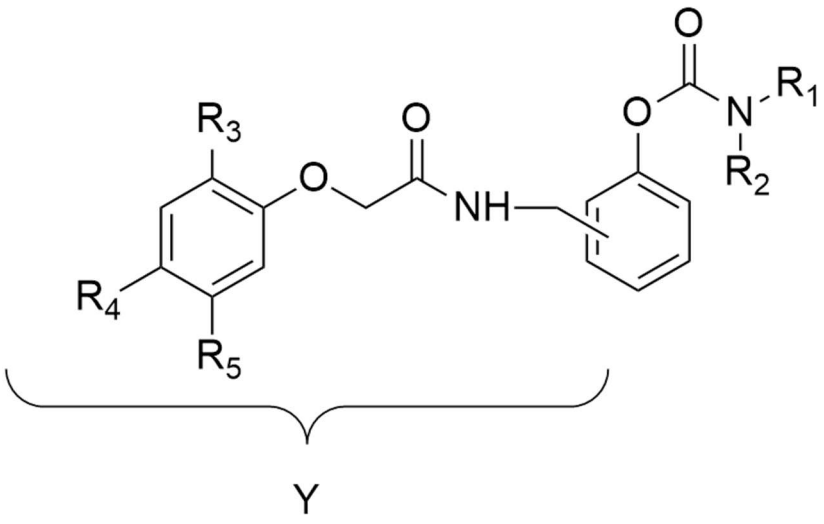
Stability assays are important to evaluate compounds for potential weaknesses at an early stage of research. In particular, plasma stability assays are a fundamental method for screening new drugs. Some enzymes can only be found in plasma, therefore in-drug development performed *in vitro* or *in vivo* can often not compare. For example, nucleases, esterases and deamidases only occur in plasma [24]. The research aim of this study is to conduct an high-throughput plasma stability assay and to generate plasma curves of the drugs. These can be used to gain insight into which of the derivatives could be used for further pharmacokinetic tests.
In addition, the stability assay was conducted in human and rat plasma to determine whether the difference in enzyme activity had a detectible effect on the DOH derivatives.

2. Methods and Materials

2.1. Chemicals

The 26 derivatives were synthesised by the team of Leonardo Brunetti and Rosalba Leuci from the University of Bari during their PhD. The structures of the compounds can be seen in Table 1. and Table 2.
The blank human plasma was purchased from VWR International (Pennsylvania, US) and Biowest (Nuaillé, France) both with citrate as anticoagulant. The blank rat plasma was purchased from NeoBiotech (Nanterre, France) with K_2-EDTA as anticoagulant.
The human and rat plasma were delivered to the University of Vienna and stored in Eppendorf tubes at -20 °C at all times.

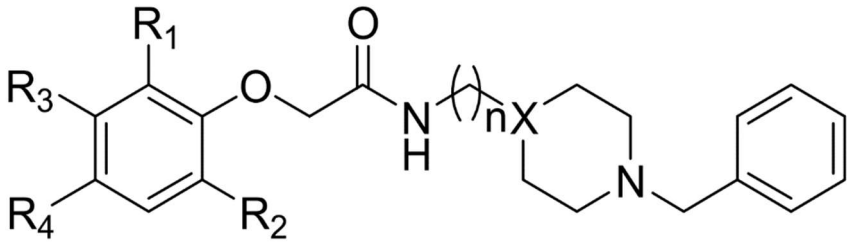
Table 1. Structures of the analysed compounds 1-23.



Compound	Y*	R_1	R_2	R_3	R_4	R_5
AN13	para	Et	Me	NO_2	Cl	H
AN15	ortho	Et	Et	H	Ph	H
GIA5	meta	Me	Me	NO_2	Cl	H
GIA13	meta	Me	Me	H	Cl	H
GIA14	meta	Me	Me	H	Ph	H
GIA17	meta	Et	Me	H	Ph	H
GIA22	meta	Et	Me	NO_2	Cl	H
GIA25	meta	Et	Me	H	Cl	H
MCC9	meta	Et	Me	NO_2	H	F
MCC13	ortho	Et	Me	H	Cl	H

MCC17	ortho	Et	Me	H	Ph	H
MCC20	ortho	Et	Me	NO_2	H	F
MCS2	para	Et	Et	H	Cl	H
ROS111	meta	Et	Et	NO_2	H	F
ROS114	para	Et	Me	H	Cl	H
ROS131	para	Et	Et	H	Ph	H
ROS150	ortho	Et	Me	NO_2	Cl	H
ROS151	meta	Me	Me	NO_2	H	F
VA2	para	Et	Et	NO_2	Cl	H
VIT8	ortho	Et	Et	NO_2	H	F
VIT10	para	Et	Et	NO_2	H	F
VIT34	para	Me	Me	H	Cl	H
VIT35	para	Me	Me	NO_2	Cl	H

Table 2. Structures of the analysed compounds 24-26.



Compound	X	n	R_1	R_2	R_3	R_4
SON38	N	2	H	NO_2	H	Cl
SON39	N	2	H	NO_2	F	H
WIN13	CH	1	H	NO_2	H	Cl

The following chemicals were purchased: DOH from Thermo Fisher ScientificTM (Gemering, Germany), formic acid (FA, 98+%) from Carl Roth GmbH + Co. KG (Karlsruhe, Germany), LiChrosolv® ACN (LC-MS grade) from Merck KGaA (Darmstadt, Germany), water with LC-MS grade from VWR International (Pennsylvania, US), HiPerSolv Chromanorm® Ultra Plus MeOH (LC-MS grade) from VWR International (Pennsylvania, US).

2.2. Equipment and materials

The scale used for weighing the analytes was a MC210 analytic balance by Sartorius Lab Instruments GmbH & Co. KG (Goettingen, Germany). For proper mixing the test tubes the shaker Vortex-Genie® (Carl Roth) was used. The plasma samples were incubated using a Thermomixer comfort by Eppendorf (Hamburg, Germany). The Thermomixer® was set to 37 °C and mix speed was set at 500 rpm. The samples were processed with the 5804 R centrifuge by Eppendorf (Hamburg, Germany).

The used UHPLC set up for measuring the various drug plasma samples was a Nexera XR ultra-high performance liquid chromatography (UHPLC) system by Shimadzu (Japan). It consists of an DGU-20A5R degassing unit, two LC- 20AD XR liquid chromatography pumps, SIL-20A XR autosampler, CTO-20AC prominence column oven, SPD-M20A prominence diode array detector (extracted chromatograms at 254 nm) and CBM-20A prominence communications bus module. During the whole time of this study the same RP-HPLC setting was used (see 2.3.). The mobile phase was generated according to the gradient, see Table 3., with acidic MeOH (0.1% FA, LC-MS grade) and acidic water (0.1% FA, LC-MS grade). For separation a 50 x 2.1 mm Kinetex® 2.6 µm PhenylHexyl with appropriate pre-column by Phenomenex® Inc. (California, US) was used. The software Lab Solutions from Shimadzu (Japan) was used for data analysis.

The used LC-HRMS consists of an Ultimate 3000 RSLC-series system by Thermo Fisher ScientificTM DionexTM (Gemering, Germany) coupled with an maXis ESI double quadrupole mass analyser TOF mass spectrometer by Bruker Corporation (Bremen, Germany). The parts of the UHPLC system were from the DionexTM UltiMate 3000 product line. This set up consists of an SRD-3400 degassing unit, RS pump, RS autosampler, RS Column Compartment, RS Diode Array Detector (set to 220 – 310nm).
Mobile and solid phase were the same as for the UHPLC as mentioned above.
The data analysation software used was the Compass DataAnalysis 4.2 by Bruker Corporation (Bremen, Germany).

2.3. UHPLC - Method setting and conditions

The RP-UHPLC conditions used for the measurements are depicted in Table 4. The solvents - solvent A = water (containing 0.1% FA, LC-MS grade), solvent B = MeOH (containing 0.1% FA, LC-MS grade) were used and mixed according to the gradient settings. The conditions were set to 0.500 mL/min flow (resulting in approximately 340 bar pump pressure), 10 µL injection volume and the column oven temperature was maintained at 37° C.

The signal-to-noise ratio (S/N ratio) was calculated by the analysing software by comparing the measured signal intensity to the background noise of the spectrogram.
To ensure the measurement integrity the two lower limits were chosen according to the ICH guidelines. None of the S/N ratios was allowed to breach below the limit of detection (LOD) = 3 and the limit of quantification (LOQ) = 10.

Table 3. Gradient used for the determination of the percentages from the compounds.

Time	Conc. H_2O [%]	Conc. MeOH [%]
0.01	80	20
1.00	80	20
7.00	5	95
10.00	5	95
10.01	80	20
13	Stop	

Every batch started with two quality control (QC) samples followed by a blank. In between the measurements the QC was measured in appropriate intervals. At the end of the batch two QC samples and a blank were measured again. The blank consists of 500 µL MeOH (0.1% FA) and 500 µL acidic water (0.1% FA). The QC sample consists of 20 µL IS and 180 µL MeOH (0.1% FA). For every batch the QC and blank samples were prepared fresh.
The consistency throughout the batch was ensured by comparing the area of the IS before and after the measurement of the samples. The deviation of the areas was not allowed to be greater than ± 15%.

Table 4. RP-UHPLC conditions used for the measurements. The solvents (Solvent A – acidic water (0.1% FA, LC-MS grade), Solvent B – acidic MeOH (0.1% FA ,LC-MS grade)) were mixed according to the gradient settings.

Item	Value	Units
Mode	Binary gradient	
Total flow	0.500	mL/min
Pressure at 0 min	340	bar
Degassing	-95	kPa
Oven temperature	37	°C
Temperature Limit	50	°C
Injection Volume	10	µL

2.4. LC-HRMS – settings and method

The LC-MS measurements were conducted with the oven temperature set to 37 °C, 0.500 mL/min flow and a decreased injection volume of 5 µL compared to the RP-UHPLC settings. The QC samples and blanks were conducted the same way as for the RP-UHPLC measurements.

2.4.1. Fragment ions of the analytes

Table 5. shows the *m/z* ratios of formed fragment ions of the derivatives as well as the molecular mass of the parent ion. In order to prevent peak overlap in the respective extracted ion chromatogram, all compounds were separated by UHPLC prior their entry into the MS. For SON39 the $[M+NH_4]^+$ adduct and for VIT34A the $[M+Na]^+$ adduct was used for analyses, due to better sensitivity. The majority of the remaining compounds were analysed by measuring their $[M+H]^+$ adduct. The compounds MCC13, MCS2 and ROS114 were in the upper limit of detection in the manner of HRMS and were thus analysed at 257 nm with the UV-Vis-detector of the LC-MS for determination of the respective degradation in human plasma instead of the $[M+H]^+$ adduct.

Table 5. A list of the molecular masses of the parent ions and the main m/z ratios of the ions formed for each of the analytes – as calculated with ChemDraw.

Analyte	Molecular mass [g/mol]	*m/z* ratio of formed ions	
AN15	432.512	433.211	$[M+H]^+$
GIA25	376.834	377.124	$[M+H]^+$
MCC13	376.834	377.125	$[M+H]^+$
MCS2	390.861	391.140	$[M+H]^+$
ROS114	376.834	377.124	$[M+H]^+$
SON38	432.901	433.162	$[M+H]^+$
SON39	398.456	417.193	$[M+NH_4]^+$
VIT8	419.404	420.155	$[M+H]^+$
VIT10	419.404	420.156	[M+H]+
VIT34A	362.807	385.091	$[M+Na]^+$
VIT35	407.805	408.094	$[M+H]^+$
WIN13	372.888	373.167	$[M+H]^+$

2.5. Stock solution preparation

The used stock solutions containing the derivatives were prepared the same way. 2 mg substance were weighed and dissolved in a 2 mL volumetric flask with DMSO. The volumetric flask was placed in the ultrasound bath for 10 minutes to attain complete dissolving. The prepared stock solutions resulted in a concentration of 1 mg/mL for the derivatives. The stock solutions were stored at -20 °C and used within a time frame of four months.

Because this was a high-throughput assay, a IS was used, and no calibration for substances was performed assuming that concentrations are in the linear range for all compounds. In addition, only a very limited amount of each compound was available to use to perform this plasma stability assay. The IS can improve precision and accuracy by minimizing volume errors. Due to the good detectability and high stability, DOH was selected as IS. 2.5 mg of DOH were weighed and dissolved in a 50 mL ACN (LCMS-grade) resulting in a concentration of 50 µg/mL. The IS was stored on ice until usage.

2.6. Incubation sample preparation

In order to determine whether the stability of the compounds is greater than that of the parent compounds, a plasma curve was prepared for each substance. To obtain the plasma curves the samples were prepared in triplicate and incubated for up to 24.00 h. The plasma curve is characterised by the plotting of time against the analytes determined percentage, resulting in a graph that can be used to approximate the $t_{1/2}$.
The plasma was taken out of the fridge and left to thaw at room temperature. After thawing 500 µL plasma were filled into 1.5 mL Eppendorf tubes. The blank plasma was vortexed and pre-warmed for 3 minutes in a Mixmate® at 37 °C to achieve better reaction once the compound was added. 50 µL of the stock solution was added to the blank plasma. The spiked plasma (SP, 90.9 µg/mL) was vortexed for 20 seconds and incubated in a Mixmate® at 37 °C for 0.00, 0.50, 1.00, 1.50, 2.00, 4.00, 24.00 h at 500 rpm.
Immediately after the spiking 40 µL of SP were taken and 100 µL ice-cold IS were added (t = 0.00 h) and the samples were vortexed. The samples were centrifuged with 14 000 rpm at 4

°C for 15 min. The supernatant fluid was transferred into vials and stored at -20 °C until analysation. Before the measurements were taken, every sample was vortexed.
All the samples were prepared in triplicate, and the workflow remained the same with the rat and human plasma.

3. Results and Discussion

3.1. Screening Experiments in plasma: Determination of time frame, percentage of analyte and *in vitro* $t_{1/2}$

The execution of the screening had to be determined depending on the amount and number of samples. With only a few compounds it is possible to use a separate Eppendorf for each incubation. A hindrance occurs when multiple compounds are to be screened. For this screening 7 samples with t=0.00 h being the starting point for precipitation, prepared in triplicate, were incubated. Thus, the incubation samples of one compound would have taken up nearly all the thermomixer slots, resulting in poor work efficiency. To achieve a more economical workflow one of the prepared triplicates contained enough spiked plasma for all points of protein precipitation.

The aim of the first screening test was to determine a time frame for the plasma stability of the derivatives. As mentioned above the *in vivo* plasma stability of DOH is approximately 80 h and of rivastigmine approximately 1 hour (see section 1.1 and 1.2). At first the incubation times were set to 0.00, 0.25, 0.50, 0.75, 1.00, 1.50, 4.00, 20.00 and 24.00 h. The enzymatic reactions were stopped by adding ice cold IS solution (preparation see section 2.5).
The two compounds MCC9 and ROS151 were incubated for 24.00 h, and samples were collected at the pre-determined incubation times. Figure *6*. and Figure *7*. depict the measured percentages of the compounds during the degradation process over the course of 24.00 h - the exact data is shown in Appendix Table 1. and Appendix Table 2.

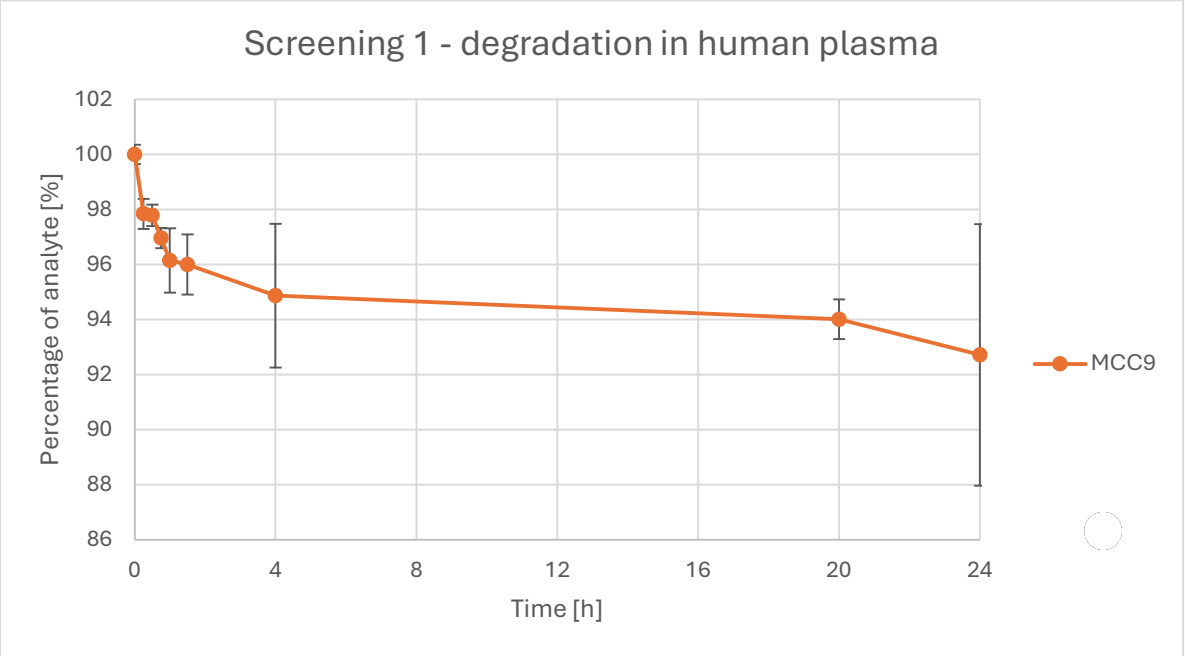


Figure 6. Degradation of incubated MCC9 in human plasma at 37 °C. The detected percentages from degradation in plasma were calculated with area(analyte)/area(IS) and are plotted against the incubation time (n=3, average ± SD). Exact data shown in Appendix Table 1. The samples were incubated for to 0.00, 0.25, 0.50, 0.75, 1.00, 1.50, 4.00, 20.00 and 24.00 h. SD = standard deviation

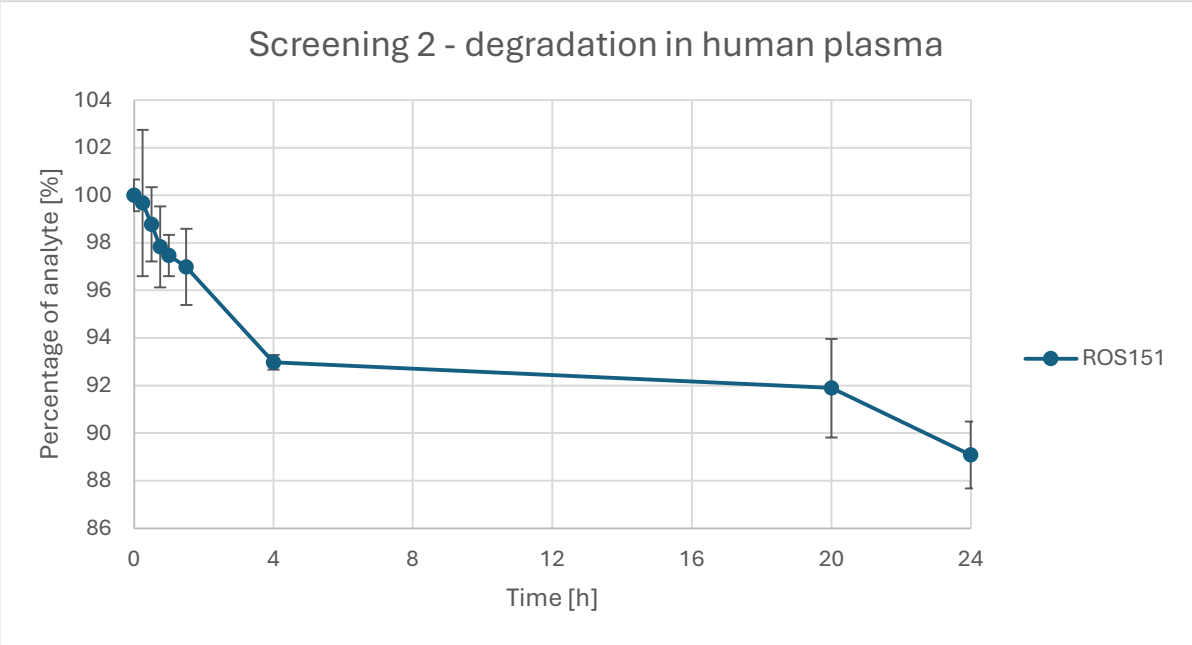


Figure 7. Degradation of incubated ROS151 in human plasma at 37 °C. The detected percentages from degradation in plasma were calculated with area(analyte)/area(IS) and are plotted against the incubation time (n=3, average ± SD). Exact data shown in Appendix Table 2. The samples were incubated for to 0.00, 0.25, 0.50, 0.75, 1.00, 1.50, 4.00, 20.00 and 24.00 h. SD = standard deviation

There was no substantial difference in degradation in between the times from 0.25 h to 1.00 h as can be seen in Figure *6*. and Figure *7*. To simplify the workflow and improve the outcome the incubation times 0.25, 0.75 and 20.00 h were eliminated, and a 2.00 h incubation time was introduced. This approach enabled the efficient utilisation of resources and the optimisation of the workflow.

With an injection volume of 40 µL at the RP-UHPLC massive peak tailing occurred. This can be explained by the fact that overload occurred due to the small particle size of the column. After experimenting with several smaller injections, the injection volume was optimised to 10 µL (data not shown).

In LC-MS measurements, the injection volume had to be modified due to oversaturation of the detector by 10 µL. The problem still occurred with 7 µL injection volume but was bypassed with the adjustment to 5 µL. Therefore, the LC-MS injection volume was optimized to 5 µL.

The percentages of the analytes were estimated by relating the peak areas of the analytes against the respective IS. For the analysis, the calculated ratio of the analytes at t = 0.00 h was assumed to be 100%. The metabolic stability was assessed by plotting the incubation time (x-axis) against the percentage of remaining analyte calculated with area(analyte)/area(IS) (y-axis). In order to calculate the *in vitro* $t^{1}/_{2}$ the incubation time was plotted against the natural logarithm of the percentage of the analyte remaining in the plasma. The linear part of the slope was used for the *in vitro* $t^{1}/_{2}$ by calculating ln2/k. The calculated values can be seen in Appendix Table 47. It has to be kept in mind that all values over 24.00 h are extrapolated.

3.2. Degradation study of AN13 in human and rat plasma

4-[(2-(4-Chloro-2-nitrophenoxy)acetamido)methyl]phenyl ethyl(methyl)carbamate, AN13 (see Figure 8.) is a DOH and rivastigmine hybrid. To determine the *in vitro* degradation rate of AN13 in human and rat plasma, samples with the same final concentration (90.9 µg/mL) were incubated at 37 °C for different time periods. The incubation was stopped with IS after 0.00, 0.50, 1.00, 1.50, 2.00, 4.00 and 24.00 h. The samples were analysed using RP-UHPLC.

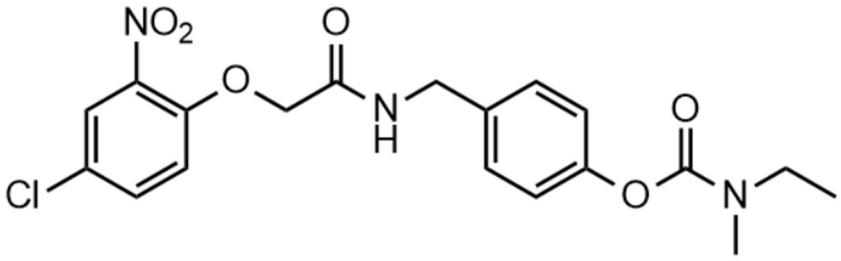


Figure 8. The structure of the DOH and rivastigmine hybrid AN13.

Figure 9. compares the results of AN13 incubation in human and rat plasma. The percentage of AN13 fell in human plasma during the time of 0.00 h to 4.00 h from 100.0±5.0% to 95.7±1.7%, as shown in Table *6*. However, in rat plasma the degradation started slower and in the first 4.00 h the percentage of the analyte sank from 100.0±6.3% to 97.1±7.6%, as can be seen in Table 7.

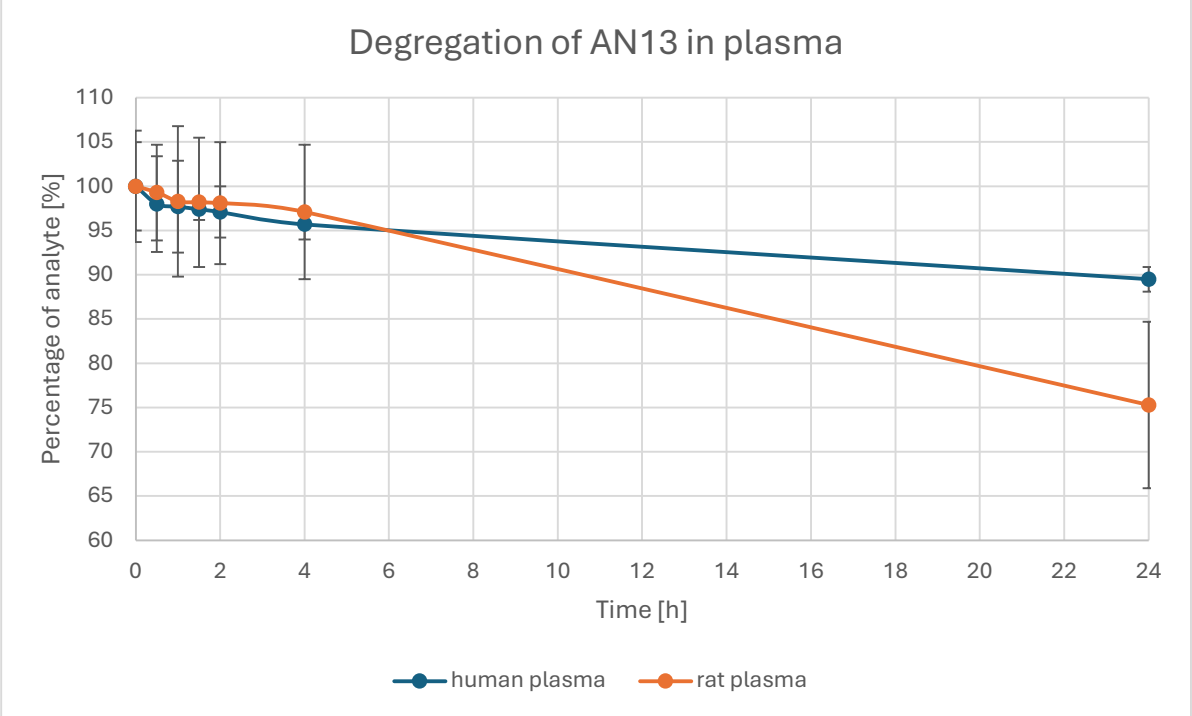
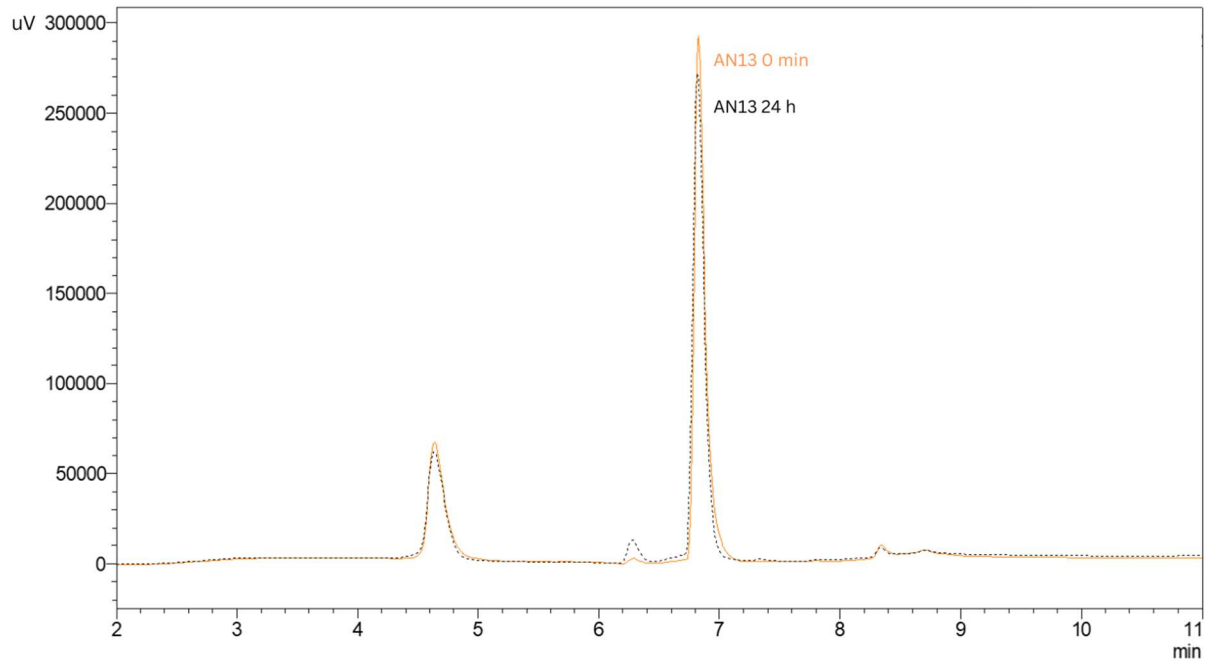


Figure 9. The results of incubating AN13 in rat and human plasma at 37°C over 24.00 h (n=3, average ± SD). The orange data shows the degradation of AN13 in human plasma, while the blue shows the degradation in rat plasma. The peak areas of the incubated samples were referenced to the respective internal standard and plotted against the incubation time. The percentages of AN13 were calculated by area(analyte)/area(IS). The individual values for AN13 in human plasma are shown in Table 6. and for rat plasma in Table 7. SD = standard deviation, IS = internal standard

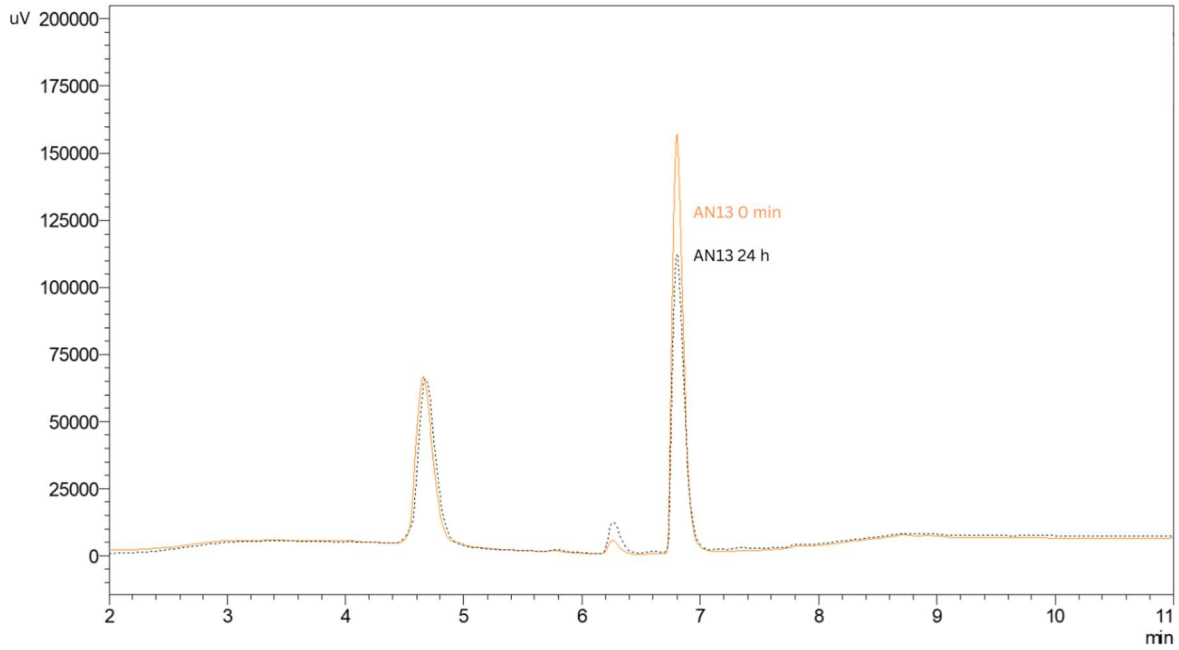
After 4.00 h there were 95.7±1.7% and 97.1±7.6% of AN13 left in human and rat plasma respectively. However, after 24.00 h of incubation, the percentage of AN13 decreased to 89.5±1.4% in human plasma and to 75.3±9.4% in rat plasma.
As displayed in Chromatogram 1., the degradation of AN13 in human plasma resulted in a relative reduction of 10,5% of the peak area between the t = 0.00 h and t = 24.00 h time points. Chromatogram 2. shows the degradation of AN13 in rat plasma after 0.00 h and 24.00 h. In rat plasma AN13 achieved a relative reduction of 24,7% during the 24-hour interval.

The $t^1/_2$ was not reached within the 24.00 h of this plasma stability experiment, neither in human, nor in rat plasma. The calculated *in vitro* $t^1/_2$ for the analyte exceeded 24.00 h in human plasma (approximately 173 h) and in rat plasma (approximately 59 h). Compared to rivastigmine, an improvement in plasma stability appears to have been achieved with this derivative. In order to ascertain whether the plasma stability of AN13 is greater than that of DOH ($t^1/_2$ = 80 h), it is necessary to conduct a plasma stability assay with a duration of at least 80 h.
The increased plasma stability can be attributed to the structural changes of AN13 compared to rivastigmine. The changes include the shift of the relative position of the ethyl(methyl)carbamate from meta to para and the addition of the 2-(4-chloro-2-nitrophenoxy)acetaldehyde moiety.

In both Chromatogram *1*. and Chromatogram *2*. a peak eluted at 6.2 min. The peak eluting at 6.2 min can be an impurity from the compound AN13 itself. This peak does not occur when injecting the blank or the IS itself. This additional peak could be a byproduct, degradation product, or leftover from the synthesis process. This impurity can be observed at t=0.00 h and increases in size until the 24.00 h mark. This could be due to enzymatic and temperature induced stress resulting in a degradation product, matching the impurity of AN13, thus increasing the size of the peak.



Chromatogram 1. Comparison between two chromatograms of AN13 incubated in human plasma measured with RP-UHPLC. The orange line shows the peak of the IS (4.6 min) and AN13 (6.8 min) after an incubation time of 0 h. The black dotted line shows the IS (4.6 min) and AN13 (6.8 min) after an incubation time of 24.00 h. The peak observed at 6.2 min can be an impurity or a degradation product of AN13. The peak area of AN13 decreased from 0.00 h to 24.00 h by 10.5%. The chromatogram depicts the running time from 2.0 – 11.0 min.



Chromatogram 2. Comparison between two chromatograms of AN13 incubated in human plasma measured with RP-UHPLC. The orange line shows the peak of the IS (4.6 min) and AN13 (6.8 min) after an incubation time of 0 h. The black dotted line shows the IS (4.6 h) and AN13 (6.8 min) after an incubation time of 24.00 h. The peak observed at 6.2 min can be an impurity or a degradation product of AN13. The peak area of AN13 decreased from 0.00 h to 24.00 h by 24,7%. The chromatogram depicts the running time from 2.0 – 11.0 min.

Table 6. Results of the incubation of AN13 in human plasma over 24.00 h. Three samples were incubated for each time and measured at 254 nm with RP-UHPLC. The detected areas of AN13 peaks were divided by the detected area of IS peaks (DOH). The average of the same time was converted to A(An)/A(IS). The calculated values were referred to the recovered AN13 peak area at t = 0.00 h to receive the percentage of the analyte AN13 (n = 3). IS – internal standard, DOH – Donepezil hydrochloride, Standard dev. – standard deviation, Var –coefficient of variation, A – peak area, An – analyte, RP-UHPLC - Reversed Phase High Performance Liquid Chromatography

	Peak area						
Time [h]	**AN13**	**Donepezil**	**A(Compound)/A(Standard)**	**A/A mean**	**Standard dev.**	**Var. [%]**	**Degradation [%]**
0	1920127	631932	3.039				
0	2041879	608252	3.357	3.209	0.160	5.0	100.0
0	1990499	616067	3.231				
0.5	1847418	625289	2.955				
0.5	2016192	613582	3.286	3.143	0.170	5.4	98.0
0.5	1938737	607942	3.189				
1	1850348	617711	2.995				
1	1891922	611295	3.095	3.135	0.163	5.2	97.7
1	1943635	586523	3.314				
1.5	1870581	594785	3.145				
1.5	1877820	609447	3.081	3.125	0.038	1.2	97.4
1.5	1895824	601847	3.150				
2	1830136	607846	3.011				
2	1904336	602066	3.163	3.115	0.090	2.9	97.1
2	1911174	602556	3.172				
4	1879078	615847	3.051				
4	1838624	606393	3.032	3.071	0.051	1.7	95.7
4	1901333	607743	3.129				
24	1753618	601943	2.913				
24	1743152	607381	2.870	2.873	0.039	1.4	89.5
24	1683638	593870	2.835				

Table 7. Results of the incubation of AN13 in rat plasma over 24.00 h. Three samples were incubated for each time and measured at 254 nm with RP-UHPLC. The detected areas of AN13 peaks were divided by the detected area of IS peaks (DOH). The average of the same time was converted to A(An)/A(IS). The calculated values were referred to the recovered AN13 peak area at t = 0.00 h to receive the percentage of the analyte AN13 (n = 3). IS – internal standard, DOH – Donepezil hydrochloride, Standard dev. – standard deviation, Var. – coefficient of variation , A – peak area, An – analyte, RP-UHPLC - Reversed Phase High Performance Liquid Chromatography

	Peak area						
Time [h]	**AN13**	**Donepezil**	**A(Compound)/A(Standard)**	**A/A mean**	**Standard dev.**	**Var. [%]**	**Degradation [%]**
0	1044992	629553	1.660				
0	1144905	624980	1.832	1.788	0.113	6.3	100.0
0	1171680	625432	1.873				
0.5	1041567	625628	1.665				
0.5	1140112	623045	1.830	1.776	0.097	5.4	99.3
0.5	1151620	627704	1.835				
1	1001292	631086	1.587				
1	1158886	633996	1.828	1.758	0.149	8.5	98.3
1	1158886	623047	1.860				
1.5	1018845	633364	1.609				
1.5	1151280	630096	1.827	1.756	0.128	7.3	98.2
1.5	1154187	629773	1.833				
2	1017739	630020	1.615				
2	1153381	633901	1.819	1.754	0.121	6.9	98.1
2	1179567	645115	1.828				
4	1010523	637656	1.585				
4	1132781	629987	1.798	1.737	0.133	7.6	97.1
4	1156797	632695	1.828				
24	912201	631229	1.445				
24	851333	612498	1.390	1.346	0.127	9.4	75.3
24	722575	600361	1.204				

3.3. Degradation study of ROS131 in human and rat plasma

4-{[2-([1,1'-Biphenyl]-4-yloxy)acetamido]methyl}phenyl diethylcarbamate, ROS131, (see Figure 10.), is a DOH and rivastigmine hybrid. To determine the *in vitro* degradation rate of ROS131 in human and rat plasma, samples with the same final concentration (90.9 µg/mL) were incubated at 37°C for different time periods. The incubation was stopped with IS after 0.00, 0.50, 1.00, 1.50, 2.00, 4.00 and 24.00 h, see Figure 11. The samples were analysed using RP-UHPLC.

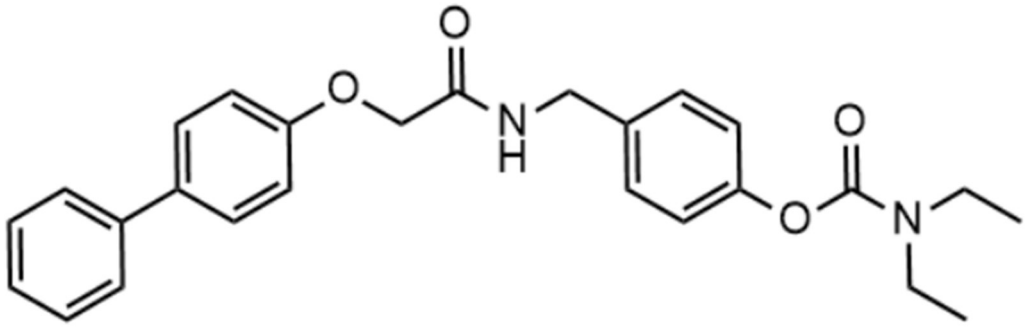


Figure 10. The structure of the DOH and rivastigmine hybrid ROS131.

In the first half hour, degradation was faster in human plasma with 97.6±2.9% than in rat plasma with 99.0±3.0%. After 1.00 h the percentage of ROS131 reached 95.4±4.5% in human and rat plasma respectively. During the first hour of incubation the level of ROS131 fell faster in rat plasma (see Figure 11.). After further incubation the degradation of ROS131 was faster in rat plasma than in human plasma. The plasma percentage of ROS131 fell to 66.4±6.3% in human plasma and 35.6±7.6% in rat plasma after 24.00 h. The exact data from the degradation studies can be found in Table *8*. and Table *9*.

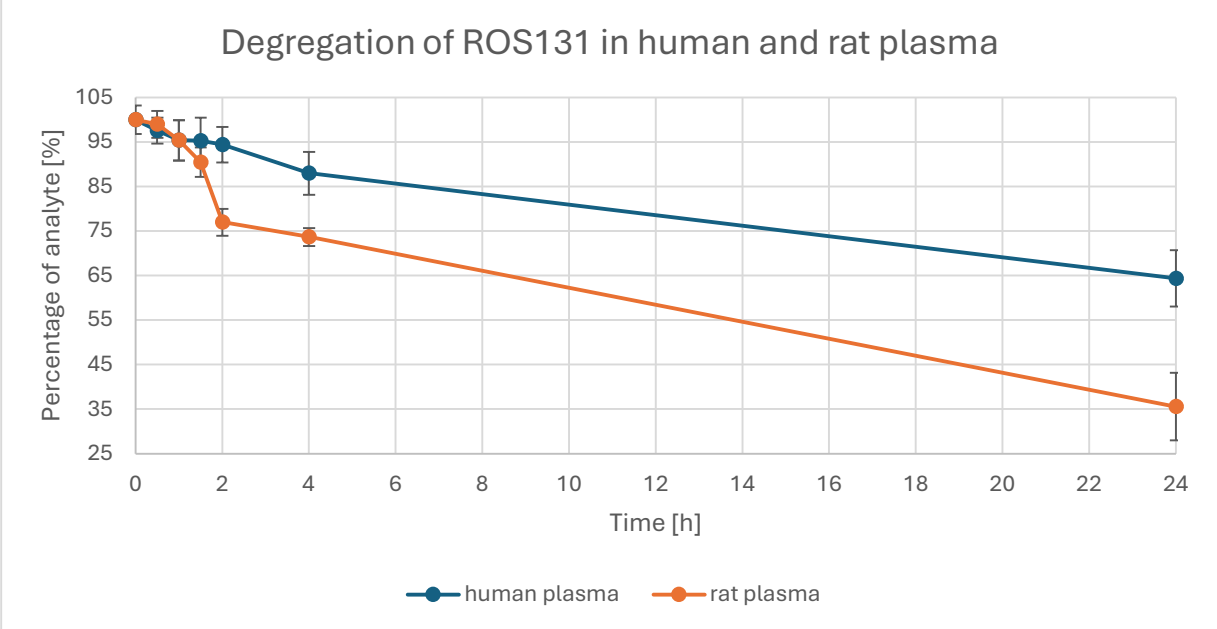


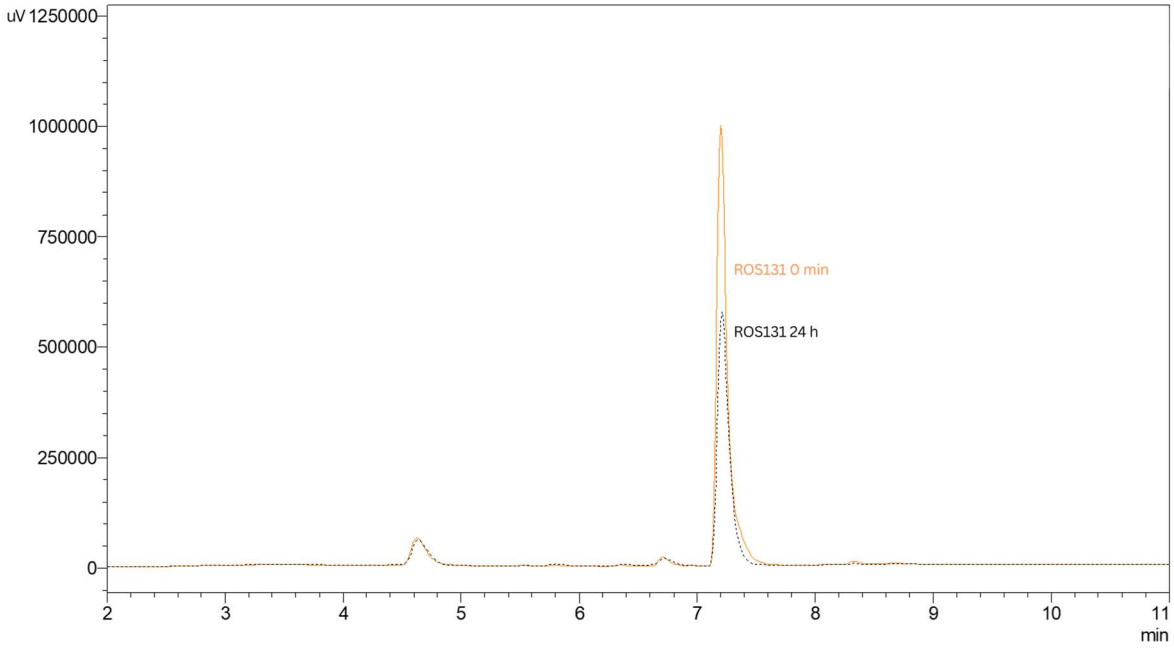
Figure 11. The results of incubating ROS131 in rat and human plasma at 37 °C over 24.00 h (n=3, average ± SD). The orange data shows the degradation of ROS131 in human plasma, while the blue shows the degradation in rat plasma. The values of the incubated samples were referenced to the respective internal standard and plotted against the incubation time. The percentages of ROS131 were calculated by area(analyte)/area(IS). The individual values for ROS 131 are shown in Table 8. and Table 9. SD = standard deviation, IS = internal standard

The degradation of ROS131 in human plasma after 0.00 h and 24.00 h can be seen in Chromatogram 3. Chromatogram 4. shows the degradation of ROS131 in rat plasma after 0.00 h and 24.00 h.

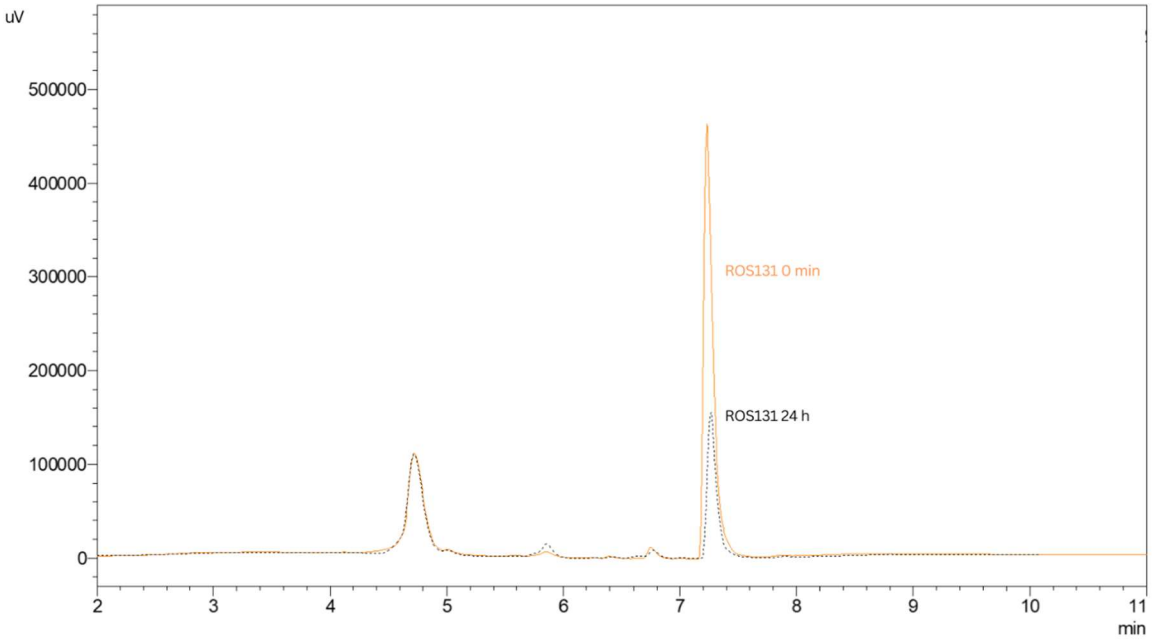
To determine the *in vitro* $t^1/_2$ of ROS131 in human plasma, longer plasma stability experiments are required. The calculated *in vitro* $t^1/_2$ for human plasma exceeded 24.00 h (approximately 39 h) and for rat plasma is 17 h. ROS131 exhibits improved plasma stability compared to rivastigmine ($t^1/_2$ = 1 h). However, to confirm whether ROS131 exhibits a indeed a lesser plasma stability than DOH, further plasma stability experiments with longer running times are necessary. Due to the $t^1/_2$ of DOH being 80 h and the calculated of $t^1/_2$ ROS131 being 39 h, the running time of the next plasma stability experiment should be at least 80 h long.

The increased plasma stability can be attributed to the structural changes of ROS131 compared to rivastigmine. One of the changes is the additional carbon atom, changing the rivastigmine moiety ethyl(methyl)carbamate to a diethylcarbamate in ROS131. Additionally, the shift of the relative position of the diethylcarbamate from meta to para was implemented. The change to the 4-{2-[(1,1'-biphenyl)-4-yloxy]acetamido}methyl moiety has presumably contributed to the change in plasma stability.
Further plasma experiments are needed to determine which enzymes cause greater degradation of ROS131 in rat plasma than in human plasma. This could be achieved by deactivating a different enzyme in each plasma stability run and comparing the results.

In the Chromatogram *4*. a peak eluted at 5.8 min after 24.00 h of incubation in rat plasma. This peak is not present in the beginning of the study (t = 0.00 h) and does not appear when injecting the blank or the IS on their own. This suggests that the peak is a degradation product of ROS131 and not a impurity in the sample itself. The formation of a peak could also be facilitated by the overall high degradation of 64.4% of ROS131 during the 24.00 h.



Chromatogram 3. Comparison between two chromatograms of ROS131 incubated in human plasma measured with RP-UHPLC. The orange line shows the peak of the IS (4.6 min) and ROS131 (7.2 min) after an incubation time of 0.00 h. The black dotted line shows the IS (4.6 min) and ROS131 (7.2 min) after an incubation time of 24.00 h. . The peak area of ROS131 decreased from 0.00 h to 24.00 h by 33.6%. The chromatogram depicts the running time from 2.0 – 11.0 min.



Chromatogram 4. Comparison between two chromatograms of ROS131 incubated in rat plasma measured with RP-UHPLC. The orange line shows the peak of the IS (4.7 h) and ROS131 (7.2 min) after an incubation time of 0.00 h. The black dotted line shows the IS (4.7 min) and ROS131 (7.2 min) after an incubation time of 24.00 h. The peak observed at 5.8 min can be an degradation product of ROS131. The peak area of ROS131 decreased from 0.00 h to 24.00 h by 64.4%. The chromatogram depicts the running time from 2.0 – 11.0 min.

Table 8. Results of the incubation of ROS131 in human plasma over 24.00 h. Three samples were incubated for each time and measured at 270 nm with RP-UHPLC. The detected areas of ROS131 peaks were divided by the detected area of IS peaks (DOH). The average of the same time was converted to A(An)/A(IS). The calculated peak areas were referred to the recovered ROS131 concentration at t = 0.00 h to receive the percentage of the analyte ROS131 (n = 3). IS – internal standard, DOH – Donepezil hydrochloride, Standard dev. – standard deviation, Var – coefficient of variation, A – peak area, An – analyte, RP-UHPLC - Reversed Phase High Performance Liquid Chromatography

	Peak area						
Time [h]	**ROS131**	**Donepezil**	**A(Compound)/A(Standard)**	**A/A mean**	**Standard dev.**	**Var. [%]**	**Degradation [%]**
0	6447495	587223	10.980				
0	6430499	591555	10.871	10.729	0.344	3.2	100.0
0	6133569	593343	10.337				
0.5	5807902	568955	10.208				
0.5	6356739	611652	10.393	10.467	0.303	2.9	97.6
0.5	6315353	584711	10.801				
1	5807902	594971	9.762				
1	6236090	583936	10.679	10.241	0.460	4.5	95.4
1	6302164	613012	10.281				
1.5	5764503	598850	9.626				
1.5	6237804	587114	10.625	10.223	0.527	5.2	95.3
1.5	6089129	584494	10.418				
2	5683296	586757	9.686				
2	6182312	590635	10.467	10.127	0.400	4.0	94.4
2	6001523	586773	10.228				
4	5401422	598011	9.032				
4	5612768	565428	9.927	9.439	0.453	4.8	88.0
4	5675892	606565	9.357				
24	4024152	583890	6.892				
24	4562704	597039	7.642	7.128	0.446	6.3	66.4
24	4022998	587442	6.848				

Table 9. Results of the incubation of ROS131 in human plasma over 24.00 h. Three samples were incubated for each time and measured at 270 nm with RP-UHPLC. The detected areas of ROS131 peaks were divided by the detected area of IS peaks (DOH). The average of the same time was converted to A(An)/A(IS). The calculated values were referred to the recovered ROS131 peak area at t = 0.00 h to receive the percentage of the analyte ROS131 (n = 3). IS – internal standard, DOH – Donepezil hydrochloride, Standard dev. – standard deviation, Var – coefficient of variation, A – peak area, An – analyte, RP-UHPLC - Reversed Phase High Performance Liquid Chromatography

	Peak area						
Time [h]	**ROS131**	**Donepezil**	**A(Compound)/A(Standard)**	**A/A mean**	**Standard dev.**	**Var. [%]**	**Degradation [%]**
0	1276881	544000	2.347				
0	2627851	1126571	2.333	2.341	0.008	0.3	100.0
0	2454100	1047248	2.343				
0.5	1478221	637800	2.318				
0.5	1513209	672956	2.249	2.318	0.070	3.0	99.0
0.5	1836997	769106	2.388				
1	2454100	1047248	2.343				
1	2237856	1012263	2.211	2.234	0.100	4.5	95.4
1	2314523	1078316	2.146				
1.5	1578470	774273	2.039				
1.5	1106434	510175	2.169	2.118	0.070	3.3	90.5
1.5	2314523	1078316	2.146				
2	1782012	948386	1.879				
2	1547371	875688	1.767	1.803	0.066	3.7	77.0
2	1500524	851110	1.763				
4	1531553	901937	1.698				
4	1500524	851110	1.763	1.725	0.034	2.0	73.7
4	1432325	836274	1.713				
24	1079621	1350387	0.799				
24	865056	1088572	0.795	0.833	0.063	7.6	35.6
24	1121812	1237985	0.906				

3.4. Degradation study of VIT8 in human and rat plasma

2-{[2-(5-Fluoro-2-nitrophenoxy)acetamido]methyl}phenyl diethylcarbamate, VIT8 (Figure 12.) is a DOH and rivastigmine hybrid. To determine the *in vitro* degradation rate of VIT8 in human and rat plasma, samples with the same final concentration (90.9 µg/mL) were incubated at 37°C for 24.00 h. The incubation was stopped with IS after 0.00, 0.50, 1.00, 1.50, 2.00, 4.00 and 24.00 h (see Figure 13.). The samples incubated in human plasma were analysed using RP-UHPLC and the samples incubated in rat plasma were analysed using MS.

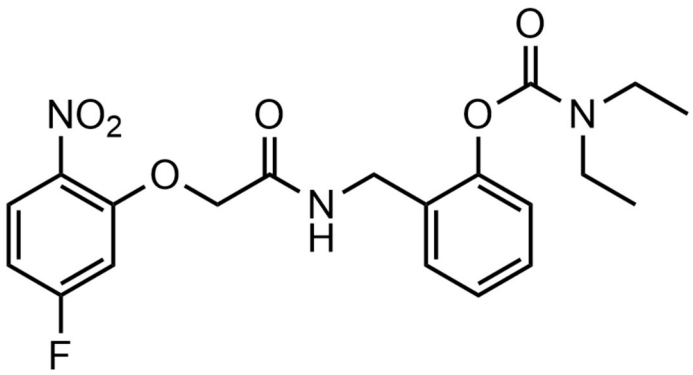


Figure 12. The structure of the DOH and rivastigmine hybrid VIT8.

Following the first half hour of incubation the percentage of the analyte VIT8 fell in human plasma to 97.1±3.2% and in rat plasma to 96.3±2.2%. Up to the two-hour mark, the percentage of the compound decreases at the same rate in human and rat plasma. In human plasma, the percentage of VIT8 gradually declines from 94.5±1.1% after two h to 68.8±5.0% after 24.00 h. Over the same timescale, there is a more pronounced decline in the degradation of the compound in rat plasma, from 87.0±3.5% to 3.5±7.8% after 24.00 h. The remaining data of the plasma studies can be seen in Table *10*. and Table *11*.

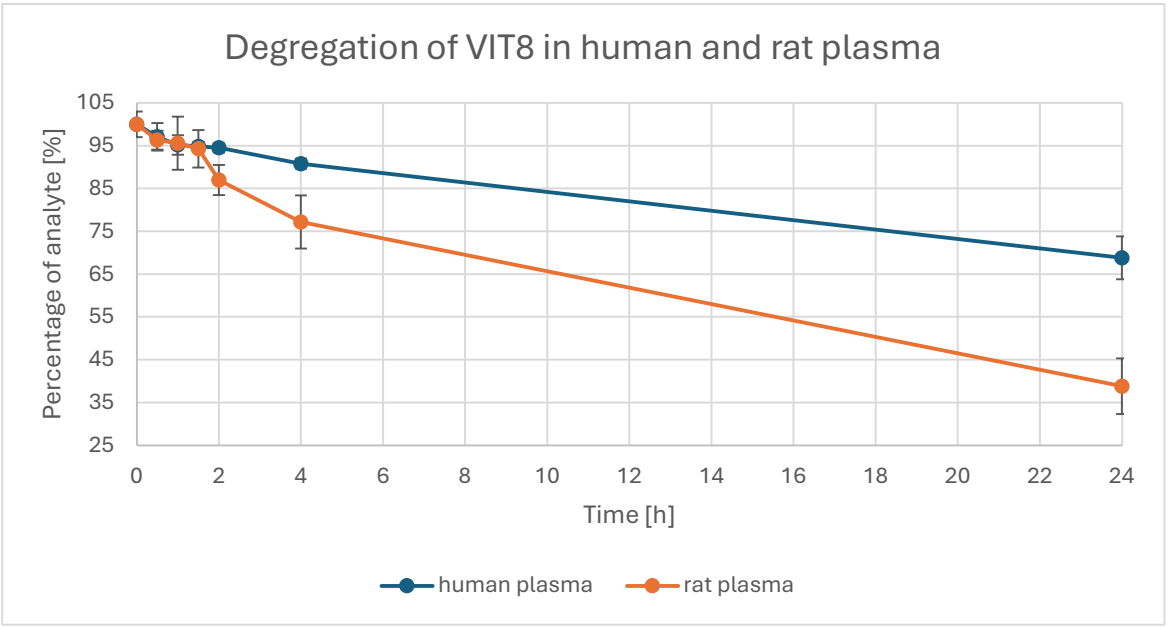


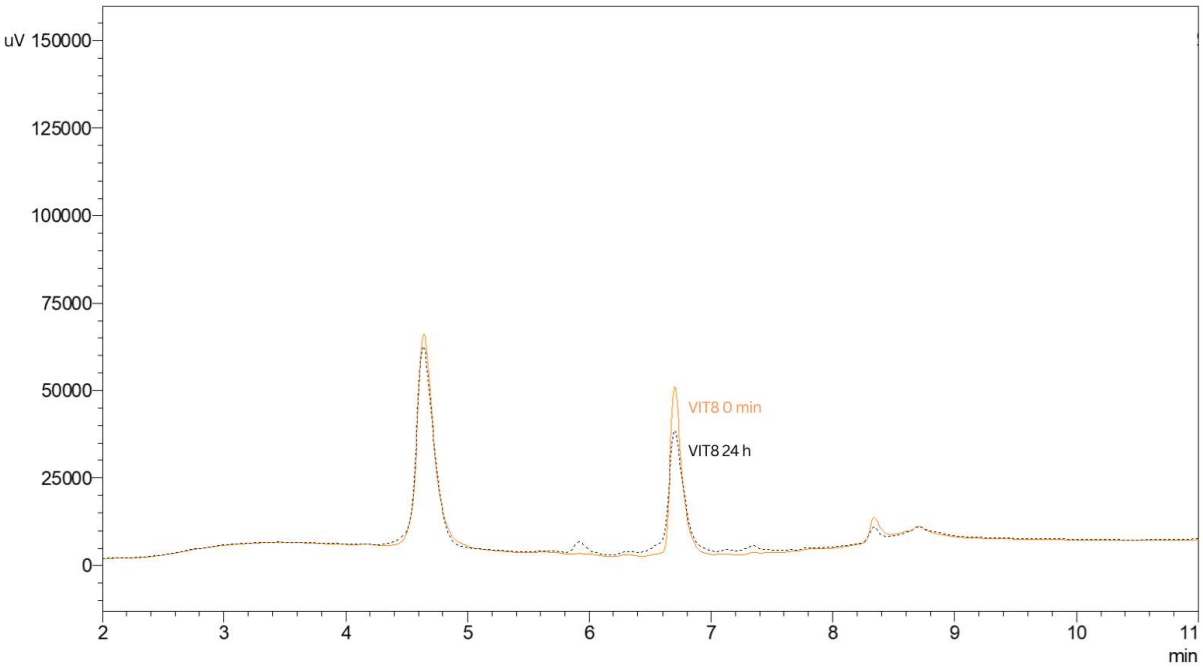
Figure 13. The results of incubating the DOH derivative VIT8 in rat and human plasma at 37 °C over 24.00 h (n=3, average ± SD). The orange data shows the degradation of VIT8 in human plasma, while the blue shows the degradation in rat plasma. The peak areas of the incubated samples were referenced to the respective internal standard and plotted against the incubation time. The percentages of VIT8 were calculated by area(analyte)/area(IS). The individual values f or VIT8 are shown in Table 10. and Table 11. DOH = donepezil hydrochloride, SD = standard deviation, IS = internal standard

The findings of the degradation study with the rivastigmine derivative VIT8 were as initially anticipated. The degradation of VIT8 was slower in human plasma than in rat plasma over the entire period observed. The degradation of VIT8 in human plasma after 0.00 h and 24.00 h can be seen in Chromatogram *5* and for rat plasma in Chromatogram *6*.

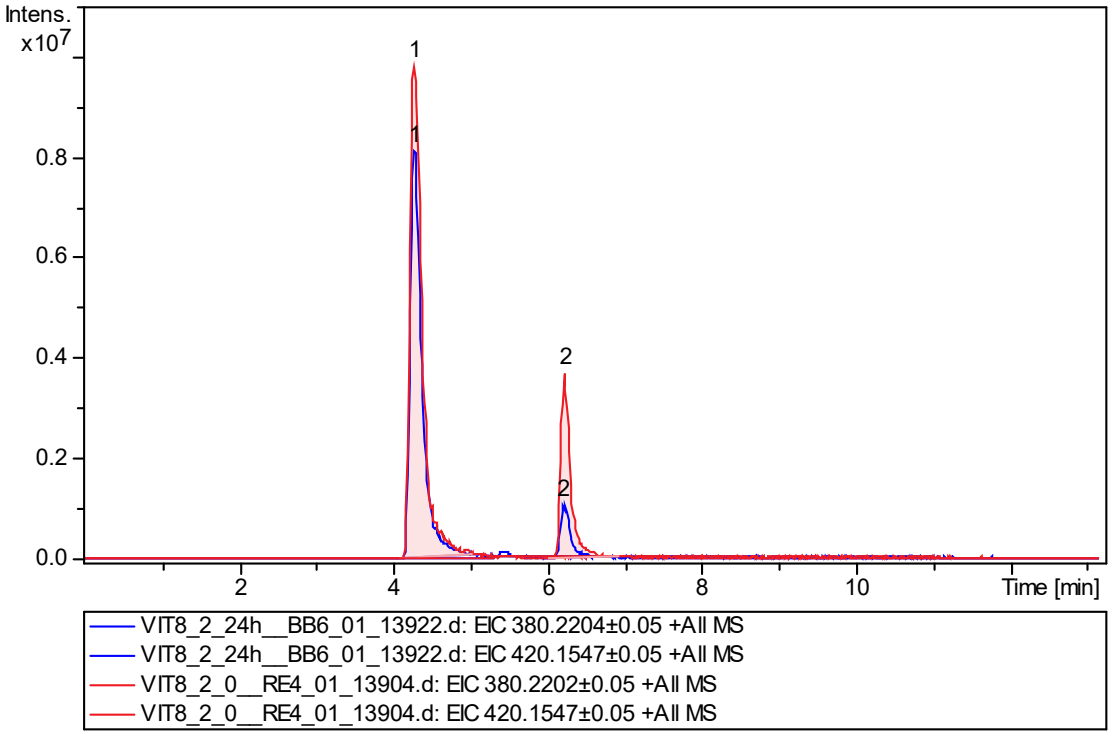
The *in vivo* $t^1/_2$ of rivastigmine in human and rat plasma is approximately 1 h. The calculated *in vitro* $t^1/_2$ of VIT8 in human plasma was found to be higher than 24.00 h (approximately 47 h) and in rat plasma 18 h. An improvement in the plasma stability of VIT8 was achieved in comparison with the parent compound rivastigmine.
VIT8 could not match the 80 h $t^1/_2$ of DOH. As the stability study was limited to 24.00 h, it was not possible to determine $t^1/_2$ of VIT8 in human plasma. To confirm, that the *in vitro* $t^1/_2$ is shorter than that of DOH, longer plasma studies need to be conducted. A plasma study with increased duration evaluating whether VIT8 demonstrates greater stability than DOH should have a minimum duration of 80 h.

The enhanced plasma stability of VIT8 can be attributed to its structural modifications relative to one of its parent compounds, rivastigmine. In the case of VIT8, the *N*-ethyl-*N*-methylcarbamate moiety was changed to a *N*-diethylcarbamate moiety. Additionally, the second rest of the phenyl was shifted from meta to ortho and replaced with the *N*-methyl-2-(5-fluoro-2-nitrophenoxy)acetamide moiety. Further plasma stability experiments are needed to determine which enzymes cause greater degradation of VIT8 in rat plasma than in human plasma.

In the Chromatogram *5.* a peak eluted at 5.9 min after 24.00 h of incubation in human plasma. This peak is not present in the beginning of the study (t = 0.00 h) and does not appear when injecting the blank or the IS on their own. This suggests that the peak is a degradation product of VIT8 and not a impurity in the sample itself.



Chromatogram 5. Comparison between two chromatograms of VIT8 incubated in human plasma measured with RP-UHPLC. The orange line shows the peak of the IS (4.6 min) and VIT8 (6.7 min) after an incubation time of 0 h. The black dotted line shows the IS (4.6 min) and VIT8 (6.7 min) after an incubation time of 24.00 h. The peak observed at 5.9 min can be a degradation product of VIT8. The peak area of VIT8 decreased from 0.00 h to 24.00 h by 31,2%. The chromatogram depicts the running time from 2.0 – 11.0 min.



Chromatogram 6. Comparison between two chromatograms of VIT8 incubated in human plasma measured with MS. The red chromatogram shows the peak of the IS (4.3 min, m/z = 380.2202±0.05) and VIT8 (6.2 min, m/z = 420.1547±0.05) after an incubation time of 0.00 h. The blue chromatogram shows the IS (4.3 min, m/z = 380.2204±0.05) and VIT8 (6.2 min, m/z = 420.1547±0.05) after an incubation time of 24.00 h. The peak area of VIT8 from 0.00 h to 24.00 h decreased by 61,2%. The chromatogram depicts the running time from 0.0 – 13.0 min.

Table 10. Results of the incubation of VIT8 in human plasma over 24.00 h. Three samples were incubated for each time and measured with MS. The detected areas of VIT8 peaks were divided by the detected area of IS peaks (DOH). The average of the same time was converted to A(An)/A(IS). The calculated values were referred to the recovered VIT8 peak area at t = 0.00 h to receive the percentage of the analyte VIT8 (n = 3). MS – Mass Spectrometry, IS – internal standard, DOH – donepezil hydrochloride, Standard dev. – standard deviation, Var – coefficient of variation, A – peak area, An – analyte

	Peak area						
Time [h]	**VIT8**	**Donepezil**	**A(Compound)/A(Standard)**	**A/A mean**	**Standard dev.**	**Var. [%]**	**Degradation [%]**
0	342310	602729	0.568				
0	342685	597784	0.573	0.570	0.003	0.5	100.0
0	338528	595647	0.568				
0.5	324553	607768	0.534				
0.5	339858	597072	0.569	0.553	0.018	3.2	97.1
0.5	336145	604566	0.556				
1	316718	598850	0.529				
1	332814	601871	0.553	0.542	0.012	2.3	95.2
1	330809	606679	0.545				
1.5	323535	593112	0.545				
1.5	312821	585866	0.534	0.540	0.006	1.1	94.8
1.5	320225	591242	0.542				
2	318847	595501	0.535				
2	318451	584141	0.545	0.538	0.006	1.1	94.5
2	312576	584803	0.534				
4	311008	601370	0.517				
4	313189	597359	0.524	0.518	0.006	1.2	90.8
4	306316	598708	0.512				
24	211000	571171	0.369				
24	237610	587275	0.405	0.392	0.019	5.0	68.8
24	237659	592014	0.401				

Table 11. Results of the incubation of VIT8 in rat plasma over 24.00 h. Three samples were incubated over 24.00 h and measured with MS. The detected areas of VIT8 peaks were divided by the detected area of IS peaks (DOH). The average of the same time was converted to A(An)/A(IS). The calculated values were referred to the recovered VIT8 peak area at t = 0.00 h to receive the percentage of the analyte VIT8 (n = 3). MS – Mass Spectrometry, IS – internal standard, DOH – Donepezil hydrochloride, Standard dev. – standard deviation, Var – coefficient of variation, A – peak area, An – analyte

	Peak area						
Time [h]	**VIT8**	**Donepezil**	**A(Compound)/A(Standard)**	**A/A mean**	**Standard dev.**	**Var. [%]**	**Degradation [%]**
0	33352490	112649232	0.296				
0	31396178	109953664	0.286	0.287	0.009	3.0	100.0
0	27521996	98623592	0.279				
0.5	35131280	130086304	0.270				
0.5	31958084	113352808	0.282	0.276	0.006	2.2	96.3
0.5	30651818	110619224	0.277				
1	28237236	108542784	0.260				
1	47699936	162658736	0.293	0.274	0.017	6.2	95.6
1	20662530	76625480	0.270				
1.5	46011444	162305648	0.283				
1.5	25050590	94011144	0.266	0.270	0.012	4.4	94.2
1.5	18627222	71393680	0.261				
2	18888502	78739144	0.240				
2	25108364	97849208	0.257	0.249	0.009	3.5	87.0
2	17612968	69928472	0.252				
4	18793472	91302376	0.206				
4	19583212	86140160	0.227	0.222	0.014	6.2	77.2
4	27286198	117799432	0.232				
24	8678500	82557888	0.105				
24	9497357	86806032	0.109	0.111	0.007	6.5	38.8
24	17902550	150073024	0.119				

3.5. Degradation study of VIT35 in human and rat plasma

4-{[2-(4-Chloro-2-nitrophenoxy)acetamido]methyl}phenyl dimethylcarbamate, VIT35, see Figure 14, is a DOH and rivastigmine hybrid. To determine the *in vitro* degradation rate of VIT35 in human and rat plasma, samples with the same final concentration (90.9 µg/mL) were incubated at 37 °C for different time periods. The incubation was stopped with IS after 0.00, 0.50, 1.00, 1.50, 2.00, 4.00 and 24.00 h, see Figure 15. The samples incubated in human plasma were analysed using RP-UHPLC and the samples incubated in rat plasma were analysed using MS.

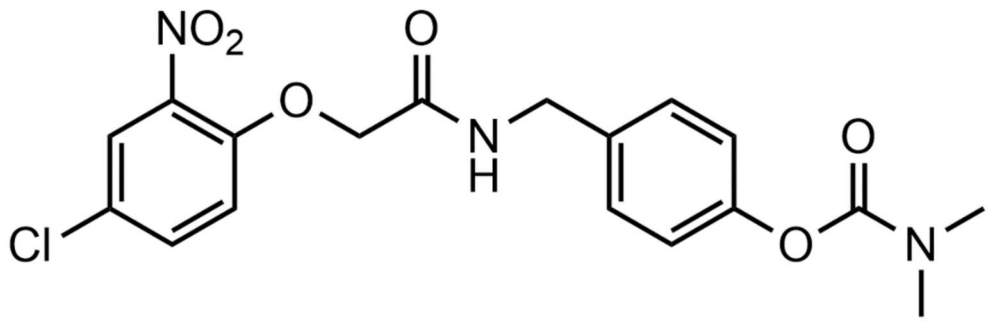


Figure 14 The structure of the DOH and rivastigmine hybrid VIT35.

The percentage of the analyte VIT35 fell in the first half hour in human plasma to 89.2±3.2%. In rat plasma the degradation was slower and VIT35 reached a plasma percentage of 97.2±3.8% in the first half hour. The degradation of VIT35 proceeded at a consistent rate in both human and rat plasma. Over the span of 24.00 h, a greater degree of degradation was observed in human plasma, with a final value of 67.9±6.5%, while in rat plasma a final percentage of 74.7±4.1% was observed. The individual values for VIT35 are shown in Table 12. for human plasma and Table 13. for rat plasma.

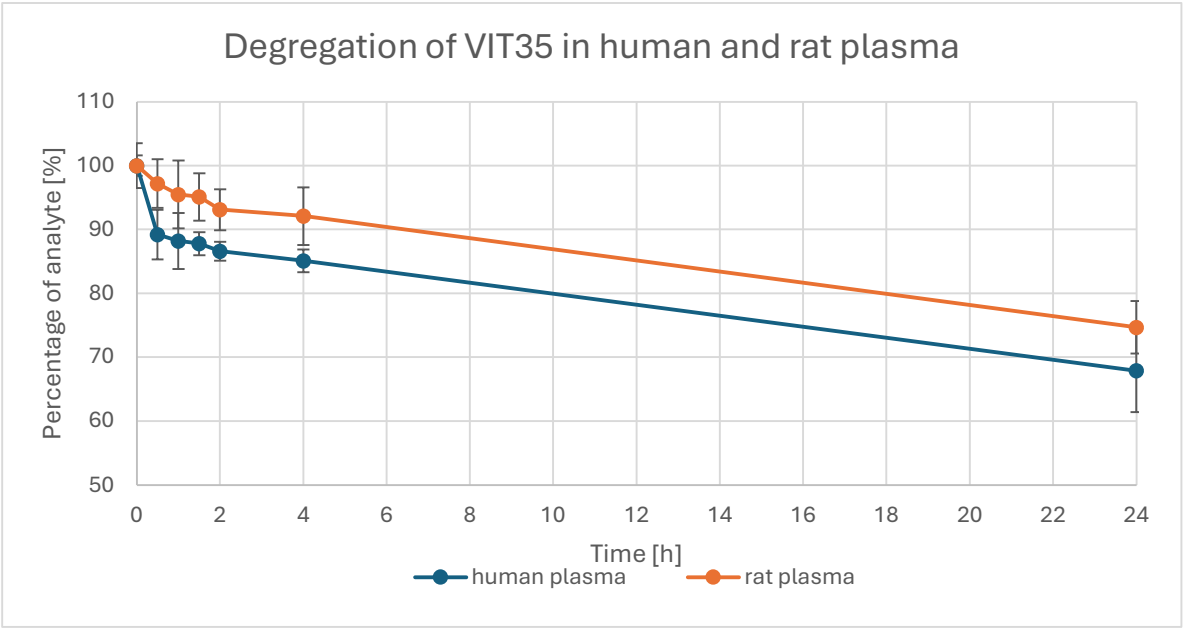


Figure 15. The results of incubating the rivastigmine derivative VIT35 in rat and human plasma at 37 °C over 24.00 h (n=3, average ± SD). The orange data shows the degradation of VIT35 in human plasma, while the blue shows the degradation in rat plasma. The peak areas of the incubated samples were referenced to the respective internal

standard and plotted against the incubation time. The percentages of VIT35 were calculated by area(a)/area(IS). The individual values for VIT35 are shown in Table 12. for human plasma and Table 13. for rat plasma. SD = standard deviation, a = analyte, IS = internal standard

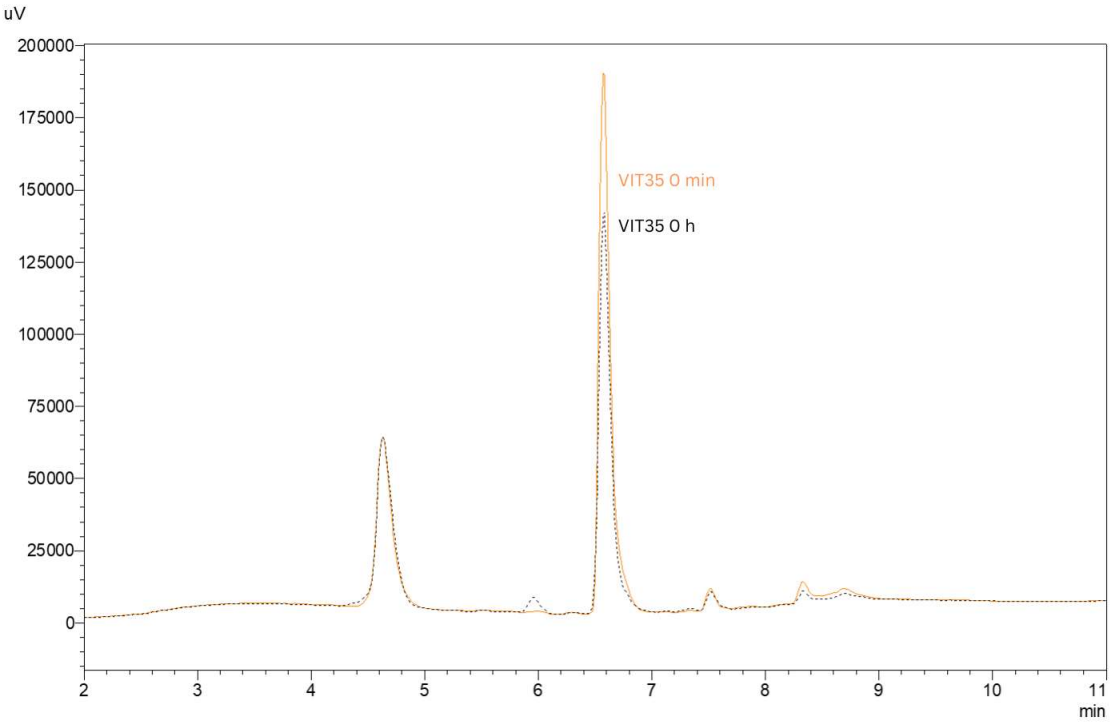
The degradation of VIT35 in human plasma after 0.00 h and 24.00 h can be seen in Chromatogram 3. Chromatogram 4. shows the degradation of VIT35 in rat plasma after 0.00 h and 24.00 h.

The calculated *in vitro* $t^{1}/_{2}$ of VIT35 exceeds 24.00 h in human plasma (approximately 55 h) and in rat plasma (approximately 62 h). Since the *in vivo* $t^{1}/_{2}$ of rivastigmine in human and rat plasma is about 1 h, an improvement in the plasma stability of VIT35 was achieved in comparison with the parent compound rivastigmine.
VIT8 could not match the 80 h $t^{1}/_{2}$ of DOH. To confirm, that the *in vitro* $t^{1}/_{2}$ is shorter than that of DOH, longer plasma studies need to be conducted. The plasma study evaluating the *in vitro* $t^{1}/_{2}$ VIT35 should have a minimum duration of 80 h.

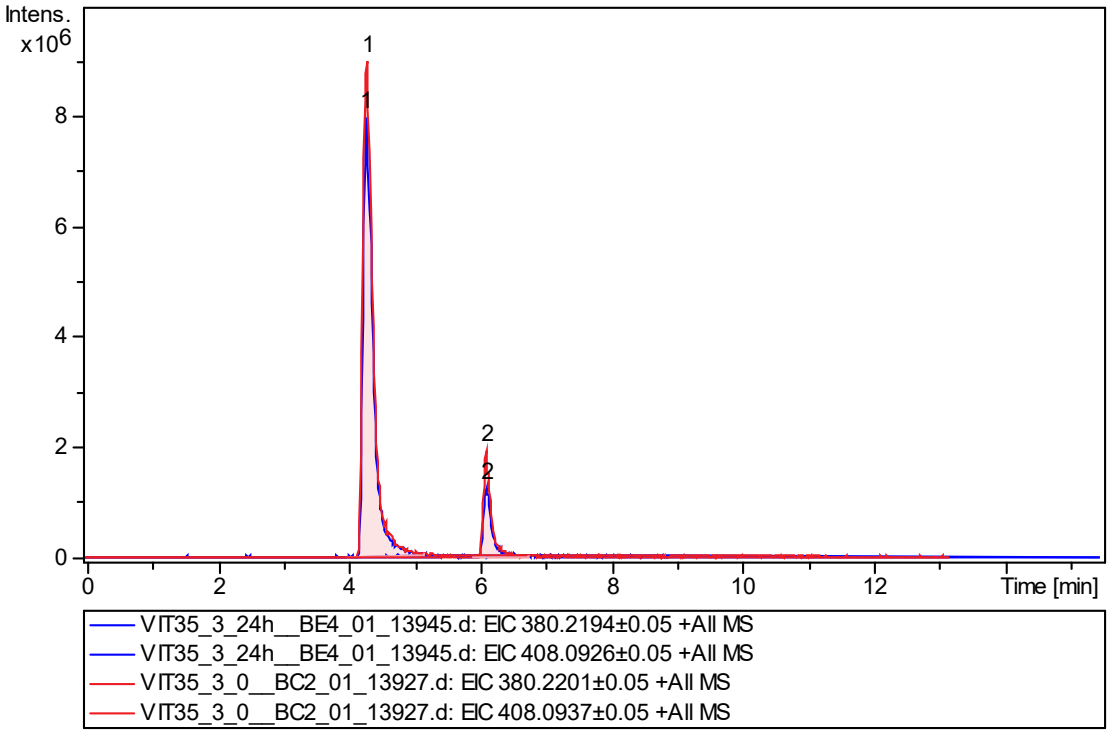
The result of this plasma stability study delivered different results than initially anticipated. The degradation of drugs is typically faster in rat plasma than in human plasma. In the degradation study of VIT35, the exact opposite occurred. Therefore, structural modifications of VIT35 must facilitate better degradation by enzymes in human plasma than in rat plasma.

In VIT35, phenyl-*N*-ethyl-*N*-methylcarbamate was modelled from rivastigmine, with the additional change from the methyl to a ethyl moiety. In addition, 2-(4-chloro-2-nitrophenoxy)-*N*-methylacetamide was added to the phenyl in para position.

In the Chromatogram 7. a peak eluted at 5.9 min after 24.00 h of incubation in human plasma. This peak is not present in the beginning of the study (t = 0.00 h) and does not appear when injecting the blank or the IS on their own. This suggests that the peak is a degradation product of VIT35 and not a impurity in the sample itself.



Chromatogram 7. Comparison between two chromatograms of VIT35 incubated in human plasma measured with RP-UHPLC. The orange line shows the peak of the IS (4.6 min) and VIT35 (7.2 min) after an incubation time of 0.00 h. The black dotted line shows the IS (4.6 min) and VIT35 (7.2 min) after an incubation time of 24.00 h. The peak observed at 5.9 min can be an degradation product of VIT35. The decrease of the peak area of VIT35 from 0.00 h to 24.00 h amounts to 32,1%. The chromatogram depicts the running time from 2.0 – 11.0 min.



Chromatogram 8. Comparison between two chromatograms of VIT35 incubated in human plasma measured with MS. The red line shows the peak of the IS (4.3 min, m/z = 380.2201±0.05) and VIT35 (6.1 min, m/z = 408.0937±0.05) after an incubation time of 0 h. The blue line shows the IS (4.3 min, m/z = 380.2194±0.05) and VIT35 (6.1 min, m/z = 408.0926±0.05)) after an incubation time of 24.00 h. The decrease of the peak area of VIT35 from 0.00 h to 24.00 h amounts to 25,3%. The chromatogram depicts the running time from 0.0 - 13.0 min.

Table 12. Results of the incubation of VIT35 in human plasma over 24.00 h. Three samples were incubated for each time and measured at 254 nm with RP-UHPLC. The detected areas of VIT35 peaks were divided by the detected area of IS peaks (DOH). The average of the same time was converted to A(An)/A(IS). The calculated values were referred to the recovered ROS131 peak area at t = 0.00 h to receive the percentage of the analyte VIT35 (n = 3). IS – internal standard, DOH – donepezil hydrochloride, Standard dev. – standard deviation, Var – coefficient of variation, A – peak area, An – analyte, RP-UHPLC - Reversed Phase High Performance Liquid Chromatography

	Peak area						
Time [h]	**VIT35**	**Donepezil**	**A(Compound)/A(Standard)**	**A/A mean**	**Standard dev.**	**Var. [%]**	**Degradation [%]**
0	1360759	584065	2.330				
0	1359624	582872	2.333	2.310	0.037	1.6	100.0
0	1325718	584753	2.267				
0.5	1211006	615039	1.969				
0.5	1268180	599213	2.116	2.060	0.080	3.9	89.2
0.5	1259626	601246	2.095				
1	1192910	616992	1.933				
1	1264948	602806	2.098	2.037	0.090	4.4	88.2
1	1254196	603223	2.079				
1.5	1193057	599701	1.989				
1.5	1185134	582031	2.036	2.029	0.036	1.8	87.8
1.5	1188914	576834	2.061				
2	1187331	603311	1.968				
2	1178193	587727	2.005	2.001	0.031	1.5	86.6
2	1188665	585846	2.029				
4	1177585	609540	1.932				
4	1175180	599781	1.959	1.965	0.036	1.8	85.1
4	1191937	595119	2.003				
24	984408	600783	1.639				
24	849985	585368	1.452	1.569	0.102	6.5	67.9
24	974860	603049	1.617				

Table 13. Results of the incubation of VIT35 in rat plasma over 24.00 h. Three samples were incubated for each time and measured with MS. The detected areas of VIT35 peaks were divided by the detected area of IS peaks (DOH). The average of the same time was converted to A(An)/A(IS). The calculated values were referred to the recovered VIT35 peak area at t = 0.00 h to receive the percentage of the analyte VIT35 (n = 3). MS – Mass Spectrometry, IS – internal standard, DOH – donepezil hydrochloride, Standard dev. – standard deviation, Var – coefficient of variation, A – peak area, An – analyte

	Peak area						
Time [h]	**VIT35**	**Donepezil**	**A(Compound)/A(Standard)**	**A/A mean**	**Standard dev.**	**Var. [%]**	**Degradation [%]**
0	27963944	156800640	0.178				
0	4746735	28333624	0.168	0.171	0.006	3.5	100.0
0	16485724	97824496	0.169				
0.5	15762470	96661368	0.163				
0.5	14171034	87081904	0.163	0.167	0.006	3.8	97.2
0.5	24611320	141475504	0.174				
1	17732706	115163208	0.154				
1	19603142	117967712	0.166	0.164	0.009	5.3	95.5
1	27706708	162066848	0.171				
1.5	11273027	71739992	0.157				
1.5	17363064	102570584	0.169	0.163	0.006	3.7	95.1
1.5	27033946	166165792	0.163				
2	16777520	109141400	0.154				
2	16756938	103223736	0.162	0.160	0.005	3.2	93.1
2	24029996	147781072	0.163				
4	15425822	103003616	0.150				
4	17087858	105636296	0.162	0.158	0.007	4.5	92.1
4	16006161	98599384	0.162				
24	6176267	49999944	0.124				
24	11561980	86361144	0.134	0.128	0.005	4.1	74.7
24	10540411	83217952	0.127				

4. Conclusion and outlook

For the most part, the expectations of the *in vitro* plasma stability experiment were met. As anticipated, there was greater degradation of the derivatives in rat plasma than in human plasma. The derivative AN13 was found to be 89.5±1.4% in human plasma and 75.3±9.4% in rat plasma, following the 24.00 h of incubation. The plasma percentage of ROS131 fell to 66.4±6.3% in human plasma and 35.6±7.6% in rat plasma after 24.00 h. With the compound VIT35 67.9±6.5 of the analyte were left in human plasma, while in rat plasma a final percentage of 74.7±4.1% was observed after 24.00 h of incubation.

However, the derivatives GIA25, ROS111 and VIT35 were an exception. For these three, the exact opposite occurred. There was an increased degradation in human plasma, as opposed to rat plasma. For example, there were 67.9±6.5% GIA25 remaining in human plasma after 24.00 h, while in rat plasma a final percentage of 74.7±4.1% was observed. Of the analyte ROS111 84.3±2.6% were left in human plasma and 89.6±3.4% in rat plasma after the incubation. Also, for VIT35 the degradation was faster in human plasma with 67.9±6.5% and 74.7±4.1% of the analyte being left in rat plasma after the 24.00 h of plasma stability experiments.
The structures of these compounds must contain certain moieties that are bound and degraded by enzymes in human plasma with a higher affinity than in rat plasma. Alternatively, it is possible that the structural changes resulted in a reduced affinity for the enzymes in rat plasma [29]. This could be due to the absence or reduced activity of the respective amide-esterase in human plasma compared to rat plasma [24]. These two hypotheses could be tested in future experiments by identifying the responsible enzymes for the fast degradation of the compounds in rat plasma, checking for their presence in human plasma and comparing the activity levels in both types of plasma.

In general, the derivatives exhibited high plasma stability, with every compound surpassing that of the parent compound rivastigmine. The analyte with the shortest calculated *in vitro* $t^1/_2$ in this stability assay was ROS131 with 39 h in human plasma and 17 h in rat plasma. With a in vivo $t^1/_2$ of approximately 1 h in human and rat plasma, ROS131 and all other compounds displayed a longer *in vitro* $t^1/_2$ than rivastigmine.
Among the calculated *in vitro* $t^1/_2$ of the derivatives, only 4 substances showed a shorter $t^1/_2$ than DOH (80 h) in human plasma. These substances were MCC13 (78 h), MCC20 (77 h), ROS131 (39 h) and VIT8 (47 h). ROS114 displayed *in vitro* in human plasma the same $t^1/_2$ as DOH *in vivo* in human plasma with 80 h. One has to keep in mind that the values exceeding 24.00 h were extrapolated.

High plasma stability can simplify working with a compound by reducing its degradation during experimental procedures. This stability can ensure that the substance remains intact under various physiological conditions, allowing for more reliable and reproducible data. Thus, leading to clearer insights into its pharmacokinetics and pharmacodynamics [25]. Enhanced stability can also reduce the complexity of analytical procedures, simplifying both *in vitro* and *in vivo* experiments. Such characteristics are particularly important in drug development, as they can improve the predictability of how the compound will behave in clinical settings [26].

Increased plasma stability often correlates with prolonged drug activity, leading to enhanced drug efficacy. If the compounds are subjected to a rapid degradation in plasma, the concentration of the drug may be insufficient to produce pharmacological activity at the therapeutic target. On the other hand, the high plasma stability of the compounds can hinder the activation of prodrugs, preventing the transformation to their active form, hindering therapeutic outcomes [19].

This was a high-throughput assay, the same workflow was performed multiple times. All 26 derivatives were measured in triplicate in human and rat plasma. Several measures can be taken to maximise the efficiency of the workflow.

The samples were placed on a MixMate in 2 mL Eppendorf tubes. After nearly half an hour into the degradation study, condensation formed in the caps of the Eppendorf tubes. Each time a sample was taken, the condensate had to be returned to the rest of the plasma by brief vortexing. When many samples are prepared, this can lead to an inconvenient extension of the work time. This additional step can easily be avoided by using a shaker mixer with an upper heat source.

Small peaks of plasma byproducts can be seen in the chromatograms. There are different measures that can be taken to avoid these. Two additional steps can be performed to increase the purity of the samples wirh this specific workflow. The sample can be collected using pipette tips with a built-in filter, which filters out the unwanted plasma byproducts, enhancing the purity of the samples. In addition, a second centrifuge run can be added to the workflow to produce a second pellet containing the plasma waste.

On the other hand, a different approach to sample purity can be taken. Solid phase extraction is the most widely used method for the removal of the biological matrix (e.g. various proteins and lipids). Solid phase extraction provides pure samples, but problems occur with the coherence of theory and the capability of prediction. Solid phase extraction is time-consuming and labour-intensive and proves itself to be more suitable for experiments with small compound quantities [42].

The next studies will assess the plasma stability of the derivatives in pig plasma and the determination of the PPB.

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6. Appendix

Appendix Table 1. Screening Experiment 1 to determine interesting time frame for the incubation of the derivatives in human plasma. Samples were prepared in triplicate, and each measured once 254 nm with RP-UHPLC. Three samples were incubated for each time and measured with MS. The detected areas of MCC9 peaks were divided by the detected area of IS peaks (DOH). The average of the same time was converted to A(An)/A(IS). The calculated values were referred to the recovered MCC9 peak area at t = 0.00 h to receive the percentage from MCC9 (n = 3). IS – internal standard, DOH – Donepezil hydrochloride, Standard dev. – standard deviation, Var – coefficient of variation, A – peak area, An – analyte, RP-UHPLC - Reversed Phase High Performance Liquid Chromatography

	Peak area						
Time [h]	**MCC9**	**Donepezil**	**A(Compound)/A(Standard)**	**A/A mean**	**Standard dev.**	**Var. [%]**	**Degradation [%]**
0	227200	984298	0.231				
0	228135	988978	0.231	0.231	0.001	0.3	100.0
0	230408	992559	0.232				
0.25	215293	946637	0.227				
0.25	221245	983620	0.225	0.226	0.001	0.6	97.8
0.25	222779	984417	0.226				
0.5	224772	998349	0.225				
0.5	224495	991999	0.226	0.226	0.001	0.4	97.8
0.5	223210	983957	0.227				
0.75	220365	986607	0.223				
0.75	222854	990364	0.225	0.224	0.001	0.4	97.0
0.75	221780	989349	0.224				
1	219474	995291	0.221				
1	223332	991363	0.225	0.222	0.003	1.2	96.1
1	221741	1002843	0.221				
1.5	218292	996028	0.219				
1.5	223690	1001549	0.223	0.222	0.002	1.1	96.0
1.5	222557	996364	0.223				
4	213090	998504	0.213				
4	219083	996614	0.220	0.219	0.006	2.6	94.9
4	221622	985758	0.225				
20	208841	1020633	0.219				
20	215835	997590	0.216	0.217	0.002	0.7	94.0
20	218095	1007194	0.217				
24	214792	997590	0.215				
24	205229	1007194	0.204	0.214	0.010	4.7	92.7
24	223299	996614	0.224				

Appendix Table 2. Screening Experiment 2 to determine interesting time frame for the incubation of the derivatives in human plasma. Samples were prepared in triplicate, and each measured once 254 nm with RP-UHPLC. Three samples were incubated for each time and measured with MS. The detected areas of ROS151 peaks were divided by the detected area of IS peaks (DOH). The average of the same time was converted to A(An)/A(IS). The calculated values were referred to the recovered ROS151 peak area at t = 0.00 h to receive the percentage of the analyte ROS151 (n = 3). MS – Mass Spectrometry, IS – internal standard, DOH – Donepezil hydrochloride, Standard dev. – standard deviation, Var – coefficient of variation, A – peak area, An – analyte, RP-UHPLC - Reverse Phase High Performance Liquid Chromatography

	Peak area						
Time [h]	**ROS151**	**Donepezil**	**A(Compound)/A(Standard)**	**A/A mean**	**Standard dev.**	**Var. [%]**	**Degradation [%]**
0	223457	986000	0.227				
0	225104	990334	0.227	0.228	0.002	0.7	100.0
0	224302	977167	0.230				
0.25	220674	938560	0.235				
0.25	216238	970920	0.223	0.227	0.007	3.1	99.7
0.25	220674	987598	0.223				
0.5	224885	985839	0.228				
0.5	220171	975156	0.226	0.225	0.004	1.6	98.8
0.5	222166	1004274	0.221				
0.75	214369	977288	0.219				
0.75	221046	993562	0.222	0.223	0.004	1.7	97.8
0.75	224706	990294	0.227				
1	218457	993704	0.220				
1	222022	994786	0.223	0.222	0.002	0.9	97.5
1	222099	995235	0.223				
1.5	218296	1004131	0.217				
1.5	219835	979418	0.224	0.221	0.004	1.6	97.0
1.5	219281	991989	0.221				
4	208069	978700	0.213				
4	208272	984760	0.211	0.212	0.001	0.3	93.0
4	223903	1059053	0.211				
20	206649	992499	0.208				
20	204542	994128	0.206	0.209	0.004	2.1	91.9
20	209178	976671	0.214				
24	196862	986000	0.200				
24	202488	990334	0.204	0.203	0.003	1.4	89.1
24	200097	977167	0.205				

Appendix Table 3. Results of the incubation of AN15 in human plasma over 24.00 h. Three samples were incubated over 24.00 h and measured at 254 nm with RP-UHPLC. The detected areas of AN15 peaks were divided by the detected area of IS peaks (DOH). The average of the same time was converted to A(An)/A(IS). The calculated values were referred to the recovered AN15 peak area at t = 0.00 h to receive the percentage of the analyte AN15 (n = 3). IS – internal standard, DOH – Donepezil hydrochloride, Standard dev. – standard deviation, Var – coefficient of variation, A – peak area, An – analyte, RP-UHPLC - Reversed Phase High Performance Liquid Chromatography

	Peak area						
Time [h]	**AN15**	**Donepezil**	**A(Compound)/A(Standard)**	**A/A mean**	**Standard dev.**	**Var. [%]**	**Degradation [%]**
0	1759065	780371	2.254				
0	1824496	771216	2.366	2.313	0.056	2.4	100.0
0	1810270	780148	2.320				
0.5	1675218	773316	2.166				
0.5	1724847	777075	2.220	2.202	0.031	1.4	95.2
0.5	1717922	774143	2.219				
1	1762125	805567	2.187				
1	1711822	783228	2.186	2.158	0.049	2.3	93.3
1	1635574	778401	2.101				
1.5	1683594	786049	2.142				
1.5	1620926	783912	2.068	2.123	0.048	2.3	91.7
1.5	1714274	794378	2.158				
2	1620074	782123	2.071				
2	1670085	767122	2.177	2.109	0.059	2.8	91.2
2	1618342	778230	2.080				
4	1620341	786018	2.061				
4	1559140	743905	2.096	2.072	0.021	1.0	89.6
4	1619491	786710	2.059				
24	1567334	803174	1.951				
24	1622057	792346	2.047	1.981	0.057	2.9	85.6
24	1560585	802251	1.945				

Appendix Table 4. Results of the incubation of AN15 in rat plasma over 24.00 h. Three samples were incubated over 24.00 h and measured with MS. The detected areas of AN15 peaks were divided by the detected area of IS peaks (DOH). The average of the same time was converted to A(An)/A(IS). The calculated values were referred to the recovered AN15 peak area at t = 0.00 h to receive the percentage of the analyte AN15 (n = 3). MS – Mass Spectrometry, IS – internal standard, DOH – Donepezil hydrochloride, Standard dev. – standard deviation, Var – coefficient of variation, A – peak area, An – analyte

	Peak area						
Time [h]	**AN15**	**Donepezil**	**A(Compound)/A(Standard)**	**A/A mean**	**Standard dev.**	**Var. [%]**	**Degradation [%]**
0	22771642	66938544	0.340				
0	23265584	66408708	0.350	0.349	0.008	2.2	100.0
0	21444368	60330944	0.355				
0.5	23209806	69580072	0.334				
0.5	23464376	69728584	0.337	0.342	0.012	3.5	98.1
0.5	28195142	79298800	0.356				
1	26462346	80419680	0.329				
1	31721118	92783408	0.342	0.337	0.007	2.1	96.7
1	22579010	66408892	0.340				
1.5	21665318	68258272	0.317				
1.5	26362684	78194392	0.337	0.328	0.010	3.0	94.0
1.5	24824858	75495384	0.329				
2	25849320	81673048	0.316				
2	26623904	80319360	0.331	0.323	0.008	2.3	92.7
2	22822432	70919320	0.322				
4	21228298	68303720	0.311				
4	26103182	79293072	0.329	0.322	0.010	3.0	92.3
4	28621140	88054208	0.325				
24	19046460	79203352	0.240				
24	17241546	71770360	0.240	0.247	0.011	4.6	70.8
24	22046508	84769072	0.260				

Appendix Table 5. Results of the incubation of GIA5 in human plasma over 24.00 h. Three samples were incubated over 24.00 h and measured at 330 nm with RP-UHPLC. The detected areas of GIA5 peaks were divided by the detected area of IS peaks (DOH). The average of the same time was converted to A(An)/A(IS). The calculated values were referred to the recovered GIA5 peak area at t = 0.00 h to receive the percentage of the analyte GIA5 (n = 3). IS – internal standard, DOH – Donepezil hydrochloride, Standard dev. – standard deviation, Var – coefficient of variation, A – peak area, An – analyte, RP-UHPLC - Reversed Phase High Performance Liquid Chromatography

	Peak area						
Time [h]	**GIA5**	**Donepezil**	**A(Compound)/A(Standard)**	**A/A mean**	**Standard dev.**	**Var. [%]**	**Degradation [%]**
0	151709	786907	0.193				
0	152421	786222	0.194	0.193	0.001	0.5	100.0
0	150478	784341	0.192				
0.5	143504	791949	0.181				
0.5	145312	801762	0.181	0.181	0.001	0.3	93.8
0.5	143702	797080	0.180				
1	141020	796257	0.177				
1	140872	794605	0.177	0.178	0.001	0.4	92.1
1	142200	797355	0.178				
1.5	139171	799032	0.174				
1.5	139226	793337	0.175	0.175	0.001	0.4	90.7
1.5	137657	787052	0.175				
2	137299	796954	0.172				
2	138530	796441	0.174	0.172	0.002	1.0	89.3
2	136335	799962	0.170				
4	137410	804092	0.171				
4	137201	802647	0.171	0.172	0.001	0.7	89.0
4	139133	804590	0.173				
24	130046	790032	0.165				
24	136773	794352	0.172	0.169	0.004	2.3	87.7
24	134544	789550	0.170				

Appendix Table 6. Results of the incubation of GIA5 in rat plasma over 24.00 h. Three samples were incubated over 24.00 h and measured at 330 nm with RP-UHPLC. The detected areas of GIA5 peaks were divided by the detected area of IS peaks (DOH). The average of the same time was converted to A(An)/A(IS). The calculated values were referred to the recovered GIA5 peak area at t = 0.00 h to receive the percentage of the analyte GIA5 (n = 3). IS – internal standard, DOH – Donepezil hydrochloride, Standard dev. – standard deviation, Var – coefficient of variation, A – peak area, An – analyte, RP-UHPLC - Reversed Phase High Performance Liquid Chromatography

	Peak area						
Time [h]	**GIA5**	**Donepezil**	**A(Compound)/A(Standard)**	**A/A mean**	**Standard dev.**	**Var. [%]**	**Degradation [%]**
0	225252	614892	0.366				
0	241562	616701	0.392	0.386	0.017	4.4	100.0
0	245156	614901	0.399				
0.5	213708	617264	0.346				
0.5	236014	614051	0.384	0.374	0.024	6.4	96.9
0.5	239577	613525	0.390				
1	210974	613858	0.344				
1	225490	618001	0.365	0.364	0.019	5.3	94.3
1	237165	620856	0.382				
1.5	214882	617645	0.348				
1.5	222330	618809	0.359	0.356	0.007	1.9	92.2
1.5	222146	617645	0.360				
2	216518	621004	0.349				
2	216727	624964	0.347	0.355	0.012	3.5	92.0
2	229357	621412	0.369				
4	222755	616854	0.348				
4	219695	613455	0.358	0.354	0.005	1.5	91.8
4	222769	626755	0.355				
24	205510	624111	0.329				
24	187651	638089	0.294	0.318	0.021	6.5	82.5
24	206350	623688	0.331				

Appendix Table 7. Results of the incubation of GIA13 in human plasma over 24.00 h. Three samples were incubated over 24.00 h and measured at 230 nm with RP-UHPLC. The detected areas of GIA13 peaks were divided by the detected area of IS peaks (DOH). The average of the same time was converted to A(An)/A(IS). The calculated values were referred to the recovered GIA13 peak area at t = 0.00 h to receive the percentage of the analyte GIA13 (n = 3). IS – internal standard, DOH – Donepezil hydrochloride, Standard dev. – standard deviation, Var – coefficient of variation, A – peak area, An – analyte, RP-UHPLC - Reversed Phase High Performance Liquid Chromatography

	Peak area						
Time [h]	**GIA13**	**Donepezil**	**A(Compound)/A(Standard)**	**A/A mean**	**Standard dev.**	**Var. [%]**	**Degradation [%]**
0	593382	2065581	0.287				
0	593576	2027828	0.293	0.291	0.004	1.2	100.0
0	601019	2045160	0.294				
0.5	587121	2055967	0.286				
0.5	589705	2070066	0.285	0.285	0.001	0.3	97.8
0.5	584354	2058385	0.284				
1	564757	2064403	0.274				
1	569506	2059134	0.277	0.275	0.002	0.6	94.3
1	561537	2049400	0.274				
1.5	549911	2090897	0.263				
1.5	571386	2092001	0.273	0.273	0.009	3.4	93.6
1.5	583715	2073352	0.282				
2	551851	2056633	0.268				
2	552656	2062989	0.268	0.270	0.002	0.9	92.5
2	558142	2049263	0.272				
4	565395	2051304	0.276				
4	541654	2082445	0.260	0.267	0.008	3.0	91.6
4	552176	2087045	0.265				
24	531236	2098833	0.253				
24	555110	2061833	0.269	0.264	0.009	3.5	90.6
24	557930	2071434	0.269				

Appendix Table 8. Results of the incubation of GIA13 in rat plasma over 24.00 h. Three samples were incubated over 24.00 h and measured at 230 nm with RP-UHPLC. The detected areas of GIA13 peaks were divided by the detected area of IS peaks (DOH). The average of the same time was converted to A(An)/A(IS). The calculated values were referred to the recovered GIA13 peak area at t = 0.00 h to receive the percentage of the analyte GIA13 (n = 3). IS – internal standard, DOH – Donepezil hydrochloride, Standard dev. – standard deviation, Var – coefficient of variation, A – peak area, An – analyte, RP-UHPLC - Reversed Phase High Performance Liquid Chromatography

	Peak area						
Time [h]	**GIA13**	**Donepezil**	**A(Compound)/A(Standard)**	**A/A mean**	**Standard dev.**	**Var. [%]**	**Degradation [%]**
0	880691	1637003	0.538				
0	922557	1606318	0.574	0.569	0.029	5.2	100.0
0	942282	1580822	0.596				
0.5	865129	1608732	0.538				
0.5	907652	1613625	0.562	0.558	0.019	3.4	98.1
0.5	901193	1567649	0.575				
1	866614	1619393	0.535				
1	905799	1580098	0.573	0.557	0.020	3.6	97.9
1	895801	1588059	0.564				
1.5	869005	1601996	0.542				
1.5	889290	1589312	0.560	0.555	0.011	2.0	97.5
1.5	902148	1599637	0.564				
2	868967	1617327	0.537				
2	879443	1618767	0.543	0.547	0.012	2.1	96.0
2	895654	1600038	0.560				
4	857009	1600943	0.535				
4	868936	1594280	0.545	0.541	0.005	1.0	95.0
4	861986	1586843	0.543				
24	845300	1634165	0.517				
24	785975	1606405	0.489	0.503	0.014	2.8	88.4
24	812734	1613236	0.504				

Appendix Table 9. Results of the incubation of GIA14 in human plasma over 24.00 h. Three samples were incubated over 24.00 h and measured at 254 nm with RP-UHPLC. The detected areas of GIA14 peaks were divided by the detected area of IS peaks (DOH). The average of the same time was converted to A(An)/A(IS). The calculated values were referred to the recovered GIA14 peak area at t = 0.00 h to receive the percentage of the analyte GIA14 (n = 3). IS – internal standard, DOH – Donepezil hydrochloride, Standard dev. – standard deviation, Var – coefficient of variation, A – peak area, An – analyte, RP-UHPLC - Reversed Phase High Performance Liquid Chromatography

	Peak area						
Time [h]	**GIA14**	**Donepezil**	**A(Compound)/A(Standard)**	**A/A mean**	**Standard dev.**	**Var. [%]**	**Degradation [%]**
0	1495877	774085	1.932				
0	1496710	772126	1.938	1.932	0.007	0.4	100.0
0	1495042	776840	1.925				
0.5	1474240	770674	1.913				
0.5	1478813	776696	1.904	1.916	0.013	0.7	99.2
0.5	1485658	769617	1.930				
1	1471859	766175	1.921				
1	1476288	777503	1.899	1.909	0.011	0.6	98.8
1	1473874	772198	1.909				
1.5	1464545	771698	1.898				
1.5	1468709	767642	1.913	1.904	0.008	0.4	98.5
1.5	1466294	771829	1.900				
2	1468476	771702	1.903				
2	1452863	773339	1.879	1.890	0.012	0.6	97.8
2	1462982	774769	1.888				
4	1456605	777056	1.875				
4	1459698	767314	1.902	1.888	0.014	0.7	97.7
4	1461676	775034	1.886				
24	1468965	778292	1.887				
24	1447198	775178	1.867	1.877	0.010	0.5	97.2
24	1455870	775178	1.878				

Appendix Table 10. Results of the incubation of GIA14 in human plasma over 24.00 h. Three samples were incubated over 24.00 h and measured at 254 nm with RP-UHPLC. The detected areas of GIA14 peaks were divided by the detected area of IS peaks (DOH). The average of the same time was converted to A(An)/A(IS). The calculated values were referred to the recovered GIA14 peak area at t = 0.00 h to receive the percentage of the analyte GIA14 (n = 3). IS – internal standard, DOH – Donepezil hydrochloride, Standard dev. – standard deviation, Var – coefficient of variation, A – peak area, An – analyte, RP-UHPLC - Reversed Phase High Performance Liquid Chromatography

	Peak area						
Time [h]	**GIA14**	**Donepezil**	**A(Compound)/A(Standard)**	**A/A mean**	**Standard dev.**	**Var. [%]**	**Degradation [%]**
0	1108070	521744	2.124				
0	1023962	483220	2.119	2.071	0.086	4.2	100.0
0	985049	499600	1.972				
0.5	990955	505323	1.961				
0.5	1060828	528912	2.006	1.986	0.023	1.1	95.9
0.5	1011733	507993	1.992				
1	1312783	667775	1.966				
1	1002328	529829	1.892	1.947	0.049	2.5	94.0
1	956708	482146	1.984				
1.5	945247	505428	1.870				
1.5	969798	502818	1.929	1.921	0.048	2.5	92.8
1.5	852481	433781	1.965				
2	717788	410946	1.747				
2	874321	469045	1.864	1.797	0.061	3.4	86.7
2	762850	428810	1.779				
4	634857	366078	1.734				
4	596403	332395	1.794	1.764	0.030	1.7	85.2
4	586201	332217	1.765				
24	1244054	769926	1.616				
24	1330201	805432	1.652	1.628	0.020	1.2	78.6
24	1293294	799502	1.618				

Appendix Table 11. Results of the incubation of GIA17 in human plasma over 24.00 h. Three samples were incubated over 24.00 h and measured at 254 nm with RP-UHPLC. The detected areas of GIA17 peaks were divided by the detected area of IS peaks (DOH). The average of the same time was converted to A(An)/A(IS). The calculated values were referred to the recovered GIA17 peak area at t = 0.00 h to receive the percentage of the analyte GIA17 (n = 3). IS – internal standard, DOH – Donepezil hydrochloride, Standard dev. – standard deviation, Var – coefficient of variation, A – peak area, An – analyte, RP-UHPLC - Reversed Phase High Performance Liquid Chromatography

	Peak area						
Time [h]	**GIA17**	**Donepezil**	**A(Compound)/A(Standard)**	**A/A mean**	**Standard dev.**	**Var. [%]**	**Degradation [%]**
0	1617781	764297	2.117				
0	1617781	772015	2.096	2.104	0.011	0.5	100.0
0	1618407	770904	2.099				
0.5	1618560	768136	2.107				
0.5	1597264	774739	2.062	2.087	0.023	1.1	99.2
0.5	1610441	769473	2.093				
1	1593833	768136	2.075				
1	1593216	768717	2.073	2.084	0.018	0.9	99.1
1	1608557	764288	2.105				
1.5	1585554	783864	2.023				
1.5	1594046	774152	2.059	2.064	0.044	2.1	98.1
1.5	1616114	765680	2.111				
2	1594427	765162	2.084				
2	1529491	793363	1.928	2.024	0.084	4.2	96.2
2	1598670	775788	2.061				
4	1578563	781935	2.019				
4	1575572	780618	2.018	2.018	0.001	0.1	95.9
4	1575875	781392	2.017				
24	1585309	801077	1.979				
24	1560504	779988	2.001	1.998	0.017	0.9	95.0
24	1573285	781392	2.013				

Appendix Table 12. Results of the incubation of GIA17 in rat plasma over 24.00 h. Three samples were incubated over 24.00 h and measured at 254 nm with RP-UHPLC. The detected areas of GIA17 peaks were divided by the detected area of IS peaks (DOH). The average of the same time was converted to A(An)/A(IS). The calculated values were referred to the recovered GIA17 peak area at t = 0.00 h to receive the percentage of the analyte GIA17 (n = 3). IS – internal standard, DOH – Donepezil hydrochloride, Standard dev. – standard deviation, Var – coefficient of variation, A – peak area, An – analyte, RP-UHPLC - Reversed Phase High Performance Liquid Chromatography

	Peak area						
Time [h]	**GIA17**	**Donepezil**	**A(Compound)/A(Standard)**	**A/A mean**	**Standard dev.**	**Var. [%]**	**Degradation [%]**
0	1463088	619173	2.363				
0	1460920	610354	2.394	2.373	0.018	0.7	100.0
0	1459795	617783	2.363				
0.5	1377540	616689	2.234				
0.5	1390917	612399	2.271	2.259	0.022	1.0	95.2
0.5	1406699	619468	2.271				
1	1391779	615089	2.263				
1	1405734	627218	2.241	2.254	0.011	0.5	95.0
1	1408159	623481	2.259				
1.5	1394938	628102	2.221				
1.5	1407009	619346	2.272	2.250	0.026	1.2	94.8
1.5	1400834	620238	2.259				
2	1394452	630020	2.213				
2	1388687	609680	2.278	2.250	0.033	1.5	94.8
2	1390562	615925	2.258				
4	1388661	625756	2.219				
4	1394323	626042	2.227	2.224	0.004	0.2	93.7
4	1394323	626277	2.226				
24	1350034	616518	2.190				
24	1356532	617709	2.196	2.191	0.004	0.2	92.3
24	1352231	617978	2.188				

Appendix Table 13. Results of the incubation of GIA22 in human plasma over 24.00 h. Three samples were incubated over 24.00 h and measured at 254 nm with RP-UHPLC. The detected areas of GIA22 peaks were divided by the detected area of IS peaks (DOH). The average of the same time was converted to A(An)/A(IS). The calculated values were referred to the recovered GIA22 peak area at t = 0.00 h to receive the percentage of the analyte GIA22 (n = 3). IS – internal standard, DOH – Donepezil hydrochloride, Standard dev. – standard deviation, Var – coefficient of variation, A – peak area, An – analyte, RP-UHPLC - Reversed Phase High Performance Liquid Chromatography

	Peak area						
Time [h]	**GIA22**	**Donepezil**	**A(Compound)/A(Standard)**	**A/A mean**	**Standard dev.**	**Var. [%]**	**Degradation [%]**
0	314646	778217	0.404				
0	319557	772918	0.413	0.407	0.005	1.3	100.0
0	314646	778217	0.404				
0.5	312269	776592	0.402				
0.5	308043	773002	0.399	0.400	0.002	0.5	98.2
0.5	310018	776644	0.399				
1	309544	774967	0.399				
1	303598	762975	0.398	0.397	0.004	0.9	97.3
1	301836	769389	0.392				
1.5	303236	779692	0.389				
1.5	304120	780682	0.390	0.391	0.002	0.6	95.9
1.5	299994	762643	0.393				
2	300640	765655	0.393				
2	298675	782909	0.381	0.385	0.006	1.6	94.6
2	293812	768517	0.382				
4	252117	680940	0.370				
4	294854	775661	0.380	0.378	0.007	1.9	92.9
4	296178	770010	0.385				
24	280032	793767	0.353				
24	295025	787182	0.375	0.364	0.011	3.0	89.4
24	291696	798135	0.365				

Appendix Table 14. Results of the incubation of GIA22 in rat plasma over 24.00 h. Three samples were incubated over 24.00 h and measured at 254 nm with RP-UHPLC. The detected areas of GIA22 peaks were divided by the detected area of IS peaks (DOH). The average of the same time was converted to A(An)/A(IS). The calculated values were referred to the recovered GIA22 peak area at t = 0.00 h to receive the percentage of the analyte GIA22 (n = 3). IS – internal standard, DOH – Donepezil hydrochloride, Standard dev. – standard deviation, Var – coefficient of variation, A – peak area, An – analyte, RP-UHPLC - Reversed Phase High Performance Liquid Chromatography

	Peak area						
Time [h]	**GIA22**	**Donepezil**	**A(Compound)/A(Standard)**	**A/A mean**	**Standard dev.**	**Var. [%]**	**Degradation [%]**
0	301656	550760	0.548				
0	304971	541354	0.563	0.553	0.009	1.6	100.0
0	276919	504757	0.549				
0.5	269119	546373	0.493				
0.5	299124	553606	0.540	0.512	0.025	4.9	92.5
0.5	277711	553250	0.502				
1	271521	558804	0.486				
1	289577	556327	0.521	0.509	0.020	3.9	91.9
1	288001	554652	0.519				
1.5	281559	558896	0.504				
1.5	273358	558015	0.490	0.496	0.007	1.4	89.7
1.5	272831	551269	0.495				
2	278445	559275	0.498				
2	274540	558381	0.492	0.493	0.005	1.0	89.0
2	270471	554073	0.488				
4	265306	561051	0.473				
4	271454	555761	0.488	0.480	0.008	1.7	86.7
4	266377	557935	0.477				
24	222895	563074	0.396				
24	239340	563741	0.425	0.414	0.016	3.7	74.8
24	236371	562308	0.420				

Appendix Table 15. Results of the incubation of GIA25 in human plasma over 24.00 h. Three samples were incubated over 24.00 h and measured at 230 nm with RP-UHPLC. The detected areas of GIA25 peaks were divided by the detected area of IS peaks (DOH). The average of the same time was converted to A(An)/A(IS). The calculated values were referred to the recovered GIA25 peak area at t = 0.00 h to receive the percentage of the analyte GIA25 (n = 3). IS – internal standard, DOH – Donepezil hydrochloride, Standard dev. – standard deviation, Var – coefficient of variation, A – peak area, An – analyte, RP-UHPLC - Reversed Phase High Performance Liquid Chromatography

	Peak area						
Time [h]	**GIA25**	**Donepezil**	**A(Compound)/A(Standard)**	**A/A mean**	**Standard dev.**	**Var. [%]**	**Degradation [%]**
0	667686	2121879	0.315				
0	715887	2096864	0.341	0.326	0.014	4.2	100.0
0	671060	2076550	0.323				
0.5	624308	1981362	0.315				
0.5	624404	1966112	0.318	0.316	0.001	0.4	96.8
0.5	631401	2003022	0.315				
1	588275	2050193	0.287				
1	646072	2002593	0.323	0.308	0.019	6.1	94.4
1	714429	2268318	0.315				
1.5	615489	1982637	0.310				
1.5	595086	2025934	0.294	0.303	0.009	2.8	92.9
1.5	609787	1994888	0.306				
2	598128	2012586	0.297				
2	592690	1994392	0.297	0.300	0.005	1.5	91.9
2	615395	2016659	0.305				
4	575972	2016614	0.286				
4	578824	2016570	0.287	0.286	0.001	0.3	87.6
4	565572	1983673	0.285				
24	568569	2038294	0.279				
24	561275	2023494	0.277	0.279	0.002	0.6	85.5
24	559268	1990867	0.281				

Appendix Table 16. Results of the incubation of GIA25 in rat plasma over 24.00 h. Three samples were incubated over 24.00 h and measured with MS. The detected areas of GIA25 peaks were divided by the detected area of IS peaks (DOH). The average of the same time was converted to A(An)/A(IS). The calculated values were referred to the recovered GIA25 peak area at t = 0.00 h to receive the percentage of the analyte GIA25 (n = 3). MS – Mass Spectrometry, IS – internal standard, DOH – Donepezil hydrochloride, Standard dev. – standard deviation, Var – coefficient of variation, A – peak area, An – analyte

	Peak area						
Time [h]	**GIA25**	**Donepezil**	**A(Compound)/A(Standard)**	**A/A mean**	**Standard dev.**	**Var. [%]**	**Degradation [%]**
0	21116316	92107648	0.229				
0	20763694	87955776	0.236	0.234	0.005	1.9	100.0
0	19867850	83513680	0.238				
0.5	26253280	111927872	0.235				
0.5	13995031	61260912	0.228	0.234	0.005	2.3	99.9
0.5	37441996	156412720	0.239				
1	22178412	95966832	0.231				
1	20212432	87925648	0.230	0.232	0.002	1.0	98.9
1	29934212	127838616	0.234				
1.5	22474708	102665928	0.219				
1.5	19935296	85066304	0.234	0.227	0.008	3.4	96.9
1.5	18733366	81987912	0.228				
2	21937700	98083936	0.224				
2	18597198	79851888	0.233	0.227	0.005	2.4	96.7
2	25659998	114994800	0.223				
4	19213510	87821416	0.219				
4	12717193	58937116	0.216	0.217	0.002	0.8	92.5
4	9266172	42871660	0.216				
24	20720884	100390728	0.206				
24	17916136	86830384	0.206	0.212	0.010	4.5	90.4
24	17552068	78762424	0.223				

Appendix Table 17. Results of the incubation of MCC9 in human plasma over 24.00 h. Three samples were incubated over 24.00 h and measured at 300 nm with RP-UHPLC. The detected areas of MCC9 peaks were divided by the detected area of IS peaks (DOH). The average of the same time was converted to A(An)/A(IS). The calculated values were referred to the recovered MCC9 peak area at t = 0.00 h to receive the percentage of the analyte MCC9 (n = 3). IS – internal standard, DOH – Donepezil hydrochloride, Standard dev. – standard deviation, Var – coefficient of variation, A – peak area, An – analyte, RP-UHPLC - Reversed Phase High Performance Liquid Chromatography

	Peak area						
Time [h]	**MCC9**	**Donepezil**	**A(Compound)/A(Standard)**	**A/A mean**	**Standard dev.**	**Var. [%]**	**Degradation [%]**
0	227200	984298	0.231				
0	228135	988978	0.231	0.231	0.001	0.3	100.0
0	230408	992559	0.232				
0.5	224772	998349	0.225				
0.5	224495	991999	0.226	0.226	0.001	0.4	97.8
0.5	223210	983957	0.227				
1	219474	995291	0.221				
1	223332	991363	0.225	0.222	0.003	1.2	96.1
1	221741	1002843	0.221				
1.5	218292	996028	0.219				
1.5	223690	1001549	0.223	0.222	0.002	1.1	96.0
1.5	222557	996364	0.223				
2	222531	996028	0.223				
2	218694	1001549	0.218	0.221	0.003	1.2	95.4
2	219098	996364	0.220				
4	213090	998504	0.213				
4	219083	996614	0.220	0.219	0.006	2.6	94.9
4	221622	985758	0.225				
24	214792	997590	0.215				
24	205229	1007194	0.204	0.214	0.010	4.7	92.7
24	223299	996614	0.224				

Appendix Table 18. Results of the incubation of MCC9 in rat plasma over 24.00 h. Three samples were incubated over 24.00 h and measured at 300 nm with RP-UHPLC. The detected areas of MCC9 peaks were divided by the detected area of IS peaks (DOH). The average of the same time was converted to A(An)/A(IS). The calculated values were referred to the recovered MCC9 peak area at t = 0.00 h to receive the percentage of the analyte MCC9 (n = 3). IS – internal standard, DOH – Donepezil hydrochloride, Standard dev. – standard deviation, Var – coefficient of variation, A – peak area, An – analyte, RP-UHPLC - Reversed Phase High Performance Liquid Chromatography

	Peak area						
Time [h]	**MCC9**	**Donepezil**	**A(Compound)/A(Standard)**	**A/A mean**	**Standard dev.**	**Var. [%]**	**Degradation [%]**
0	242431	771840	0.314				
0	240121	782373	0.307	0.311	0.004	1.2	100.0
0	241518	775129	0.312				
0.5	228543	776198	0.294				
0.5	237542	769241	0.309	0.303	0.008	2.6	97.6
0.5	237634	773920	0.307				
1	228362	782392	0.292				
1	230202	780456	0.295	0.294	0.002	0.8	94.7
1	229707	774416	0.297				
1.5	222299	797741	0.279				
1.5	230026	770497	0.299	0.294	0.013	4.5	94.5
1.5	236351	777542	0.304				
2	228685	785871	0.291				
2	229287	773148	0.297	0.292	0.004	1.5	93.9
2	225415	782021	0.288				
4	225119	781986	0.288				
4	224862	775659	0.290	0.288	0.001	0.5	92.7
4	223001	776691	0.287				
24	157085	762196	0.206				
24	155488	764234	0.203	0.205	0.002	0.8	66.1
24	158491	766609	0.207				

Appendix Table 19. Results of the incubation of MCC13 in human plasma over 24.00 h. Three samples were incubated over 24.00 h and measured with the uV-detector of MS. The detected areas of MCC13 peaks were divided by the detected area of IS peaks (DOH). The average of the same time was converted to A(An)/A(IS). The calculated values were referred to the recovered MCC13 peak area at t = 0.00 h to receive the percentage of the analyte MCC13 (n = 3). MS – Mass Spectrometry, IS – internal standard, DOH – Donepezil hydrochloride, Standard dev. – standard deviation, Var – coefficient of variation, A – peak area, An – analyte

	Peak area						
Time [h]	**MCC13**	**Donepezil**	**A(Compound)/A(Standard)**	**A/A mean**	**Standard dev.**	**Var. [%]**	**Degradation [%]**
0	54346	102636	0.530				
0	56021	109747	0.510	0.516	0.012	2.2	100.0
0	57183	112400	0.509				
0.5	57333	116661	0.491				
0.5	57004	112543	0.507	0.501	0.008	1.7	97.1
0.5	56604	112071	0.505				
1	54525	110366	0.494				
1	56745	112333	0.505	0.499	0.006	1.1	96.7
1	56524	113510	0.498				
1.5	55284	116541	0.474				
1.5	56231	114940	0.489	0.489	0.014	2.9	94.6
1.5	57075	113642	0.502				
2	57545	119780	0.480				
2	54478	117275	0.465	0.483	0.020	4.2	93.6
2	59693	118318	0.505				
4	55894	121908	0.458				
4	53235	110209	0.483	0.475	0.015	3.1	92.1
4	57433	118478	0.485				
24	48083	117421	0.409				
24	51668	123682	0.418	0.406	0.014	3.3	78.7
24	45065	115171	0.391				

Appendix Table 20. Results of the incubation of MCC13 in rat plasma over 24.00 h. Three samples were incubated over 24.00 h and measured with MS. The detected areas of MCC13 peaks were divided by the detected area of IS peaks (DOH). The average of the same time was converted to A(An)/A(IS). The calculated values were referred to the recovered MCC13 peak area at t = 0.00 h to receive the percentage of the analyte MCC13 (n = 3). MS – Mass Spectrometry, IS – internal standard, DOH – Donepezil hydrochloride, Standard dev. – standard deviation, Var – coefficient of variation, A – peak area, An – analyte

	Peak area						
Time [h]	**MCC13**	**Donepezil**	**A(Compound)/A(Standard)**	**A/A mean**	**Standard dev.**	**Var. [%]**	**Degradation [%]**
0	12031054	49579548	0.243				
0	16251517	65025692	0.250	0.243	0.007	2.7	100.0
0	16485525	69646096	0.237				
0.5	18538576	82326952	0.225				
0.5	18463456	77191392	0.239	0.236	0.010	4.2	97.2
0.5	20449690	83632536	0.245				
1	11644319	52834672	0.220				
1	17327172	71364840	0.243	0.231	0.011	4.9	95.1
1	18784018	81537296	0.230				
1.5	20596586	90603160	0.227				
1.5	20867290	92951144	0.224	0.227	0.002	0.9	93.3
1.5	19643460	86057336	0.228				
2	17519630	78730856	0.223				
2	1963704	8744780	0.225	0.222	0.003	1.3	91.3
2	15369990	70204568	0.219				
4	18164766	83566816	0.217				
4	8639570	42467244	0.203	0.210	0.007	3.3	86.5
4	19641284	93627912	0.210				
24	16194805	96654352	0.168				
24	18785798	98422056	0.191	0.177	0.012	7.1	72.7
24	13299032	77519680	0.172				

Appendix Table 21. Results of the incubation of MCC17 in human plasma over 24.00 h. Three samples were incubated over 24.00 h and measured at 254 nm with RP-UHPLC. The detected areas of MCC17 peaks were divided by the detected area of IS peaks (DOH). The average of the same time was converted to A(An)/A(IS). The calculated values were referred to the recovered MCC17 peak area at t = 0.00 h to receive the percentage of the analyte MCC17 (n = 3). IS – internal standard, DOH – Donepezil hydrochloride, Standard dev. – standard deviation, Var – coefficient of variation, A – peak area, An – analyte, RP-UHPLC - Reversed Phase High Performance Liquid Chromatography

	Peak area						
Time [h]	**MCC17**	**Donepezil**	**A(Compound)/A(Standard)**	**A/A mean**	**Standard dev.**	**Var. [%]**	**Degradation [%]**
0	1535637	767822	2.000				
0	1654010	781849	2.116	2.059	0.058	2.8	100.0
0	1608069	780003	2.062				
0.5	1553694	788014	1.972				
0.5	1621590	780196	2.078	2.047	0.065	3.2	99.4
0.5	1614579	772281	2.091				
1	1538339	780653	1.971				
1	1610470	782173	2.059	2.032	0.053	2.6	98.7
1	1604552	776746	2.066				
1.5	1500571	785777	1.910				
1.5	1592822	770855	2.066	2.015	0.091	4.5	97.9
1.5	1594351	770339	2.070				
2	1490715	785295	1.898				
2	1578817	767471	2.057	2.011	0.098	4.9	97.7
2	1593689	767365	2.077				
4	1520039	769850	1.974				
4	1539575	756475	2.035	1.993	0.037	1.8	96.8
4	1504327	764087	1.969				
24	1511997	777516	1.945				
24	1502773	777852	1.932	1.966	0.049	2.5	95.5
24	1535389	759398	2.022				

Appendix Table 22. Results of the incubation of MCC17 in human plasma over 24.00 h. Three samples were incubated over 24.00 h and measured at 254 nm with RP-UHPLC. The detected areas of MCC17 peaks were divided by the detected area of IS peaks (DOH). The average of the same time was converted to A(An)/A(IS). The calculated values were referred to the recovered MCC17 peak area at t = 0.00 h to receive the percentage of the analyte MCC17 (n = 3). IS – internal standard, DOH – Donepezil hydrochloride, Standard dev. – standard deviation, Var – coefficient of variation, A – peak area, An – analyte, RP-UHPLC - Reversed Phase High Performance Liquid Chromatography

	Peak area						
Time [h]	**MCC17**	**Donepezil**	**A(Compound)/A(Standard)**	**A/A mean**	**Standard dev.**	**Var. [%]**	**Degradation [%]**
0	1518073	609529	2.491				
0	1513350	597927	2.531	2.534	0.045	1.8	100.0
0	1580594	612399	2.581				
0.5	1467597	601331	2.441				
0.5	1462986	605138	2.418	2.493	0.111	4.5	98.4
0.5	1585940	605202	2.621				
1	1433177	606056	2.365				
1	1465222	623306	2.351	2.438	0.139	5.7	96.2
1	1610752	619977	2.598				
1.5	1565598	632726	2.474				
1.5	1442082	627630	2.298	2.423	0.109	4.5	95.6
1.5	1551044	621321	2.496				
2	1400401	568266	2.464				
2	1473279	639634	2.303	2.419	0.101	4.2	95.5
2	1583104	635754	2.490				
4	1429078	639971	2.233				
4	1469716	625267	2.351	2.357	0.127	5.4	93.0
4	1593448	640605	2.487				
24	1404952	774216	1.815				
24	1255867	723685	1.735	1.761	0.046	2.6	69.5
24	1318968	760578	1.734				

Appendix Table 23. Results of the incubation of MCC20 in human plasma over 24.00 h. Three samples were incubated over 24.00 h and measured at 254 nm with RP-UHPLC. The detected areas of MCC20 peaks were divided by the detected area of IS peaks (DOH). The average of the same time was converted to A(An)/A(IS). The calculated values were referred to the recovered MCC20 peak area at t = 0.00 h to receive the percentage of the analyte MCC20 (n = 3). IS – internal standard, DOH – Donepezil hydrochloride, Standard dev. – standard deviation, Var – coefficient of variation, A – peak area, An – analyte, RP-UHPLC - Reversed Phase High Performance Liquid Chromatography

	Peak area						
Time [h]	**MCC20**	**Donepezil**	**A(Compound)/A(Standard)**	**A/A mean**	**Standard dev.**	**Var. [%]**	**Degradation [%]**
0	286089	796217	0.359				
0	318458	797364	0.399	0.376	0.021	5.5	100.0
0	294176	794726	0.370				
0.5	287192	789368	0.364				
0.5	294192	783061	0.376	0.374	0.010	2.6	99.5
0.5	297442	775947	0.383				
1	290136	775173	0.374				
1	286456	772142	0.371	0.374	0.002	0.7	99.3
1	287791	765700	0.376				
1.5	286355	782795	0.366				
1.5	291583	774176	0.377	0.372	0.005	1.5	98.8
1.5	288121	773507	0.372				
2	279622	794730	0.352				
2	290239	786015	0.369	0.364	0.011	3.0	96.8
2	287971	774348	0.372				
4	262201	780779	0.336				
4	286870	775989	0.370	0.355	0.017	4.9	94.3
4	274807	764966	0.359				
24	222221	780009	0.285				
24	232491	770385	0.302	0.303	0.018	6.0	80.4
24	249070	776194	0.321				

Appendix Table 24. Results of the incubation of MCC20 in rat plasma over 24.00 h. Three samples were incubated over 24.00 h and measured at 254 nm with RP-UHPLC. The detected areas of MCC20 peaks were divided by the detected area of IS peaks (DOH). The average of the same time was converted to A(An)/A(IS). The calculated values were referred to the recovered MCC20 peak area at t = 0.00 h to receive the percentage of the analyte MCC20 (n = 3). IS – internal standard, DOH – Donepezil hydrochloride, Standard dev. – standard deviation, Var – coefficient of variation, A – peak area, An – analyte, RP-UHPLC - Reversed Phase High Performance Liquid Chromatography

	Peak area						
Time [h]	**MCC20**	**Donepezil**	**A(Compound)/A(Standard)**	**A/A mean**	**Standard dev.**	**Var. [%]**	**Degradation [%]**
0	281476	632573	0.445				
0	304679	629697	0.484	0.466	0.020	4.2	100.0
0	294101	627944	0.468				
0.5	267479	635067	0.421				
0.5	288233	627622	0.459	0.442	0.019	4.4	95.0
0.5	284392	637017	0.446				
1	261767	631108	0.415				
1	278088	638527	0.436	0.434	0.019	4.3	93.2
1	286920	635178	0.452				
1.5	260153	634787	0.410				
1.5	269793	630279	0.428	0.417	0.010	2.3	89.5
1.5	261821	634103	0.413				
2	257077	635241	0.405				
2	262997	631473	0.416	0.413	0.007	1.8	88.7
2	265233	634388	0.418				
4	237562	633140	0.375				
4	251473	631434	0.398	0.386	0.012	3.0	82.8
4	242728	633544	0.383				
24	173825	571894	0.304				
24	199440	644694	0.309	0.301	0.010	3.3	64.6
24	182679	630244	0.290				

Appendix Table 25. Results of the incubation of MCS2 in human plasma over 24.00 h. Three samples were incubated over 24.00 h and measured with the uV-detector of MS. The detected areas of MCS2 peaks were divided by the detected area of IS peaks (DOH). The average of the same time was converted to A(An)/A(IS). The calculated values were referred to the recovered MCS2 peak area at t = 0.00 h to receive the percentage of the analyte MCS2 (n = 3). MS – Mass Spectrometry, IS – internal standard, DOH – Donepezil hydrochloride, Standard dev. – standard deviation, Var – coefficient of variation, A – peak area, An – analyte

	Peak area						
Time [h]	**MCS2**	**Donepezil**	**A(Compound)/A(Standard)**	**A/A mean**	**Standard dev.**	**Var. [%]**	**Degradation [%]**
0	21793138	81934936	0.266				
0	21010346	82737640	0.254	0.260	0.006	2.3	100.0
0	23064940	88746288	0.260				
0.5	3682101	15028836	0.245				
0.5	9768026	38698344	0.252	0.252	0.007	2.7	97.0
0.5	17835202	68900824	0.259				
1	21734922	86675800	0.251				
1	5609492	22911904	0.245	0.252	0.007	2.9	96.8
1	22335444	86133952	0.259				
1.5	12731594	50864644	0.250				
1.5	21627354	89755024	0.241	0.243	0.007	2.9	93.3
1.5	22896130	96842096	0.236				
2	19222306	81604280	0.236				
2	20765072	85753048	0.242	0.242	0.006	2.6	93.1
2	20614758	83129896	0.248				
4	21246038	89185304	0.238				
4	19933116	83608024	0.238	0.242	0.006	2.4	92.9
4	23329210	93994648	0.248				
24	21749920	92402648	0.235				
24	17279060	75305040	0.229	0.230	0.005	2.0	88.6
24	22115310	97750744	0.226				

Appendix Table 26. Results of the incubation of MCS2 in rat plasma over 24.00 h. Three samples were incubated over 24.00 h and measured with MS. The detected areas of MCS2 peaks were divided by the detected area of IS peaks (DOH). The average of the same time was converted to A(An)/A(IS). The calculated values were referred to the recovered MCS2 peak area at t = 0.00 h to receive the percentage of the analyte MCS2 (n = 3). MS – Mass Spectrometry, IS – internal standard, DOH – Donepezil hydrochloride, Standard dev. – standard deviation, Var – coefficient of variation, A – peak area, An – analyte

	Peak area						
Time [h]	**MCS2**	**Donepezil**	**A(Compound)/A(Standard)**	**A/A mean**	**Standard dev.**	**Var. [%]**	**Degradation [%]**
0	15435392	65114580	0.237				
0	13184982	58422140	0.226	0.235	0.008	3.6	100.0
0	16250513	67084128	0.242				
0.5	18760516	84127312	0.223				
0.5	17766972	74751824	0.238	0.233	0.008	3.6	99.0
0.5	16922816	71278288	0.237				
1	17171378	75094320	0.229				
1	12742263	56035000	0.227	0.231	0.006	2.4	98.4
1	20089092	84548144	0.238				
1.5	18365728	83501064	0.220				
1.5	19504178	86124600	0.226	0.225	0.004	1.8	95.6
1.5	18850552	82846496	0.228				
2	18203932	84258448	0.216				
2	17281500	75227200	0.230	0.225	0.007	3.3	95.5
2	16208138	71156840	0.228				
4	18573360	83696056	0.222				
4	19182406	87461600	0.219	0.222	0.003	1.3	94.5
4	21365794	95000792	0.225				
24	17197028	84064648	0.205				
24	21473764	99728168	0.215	0.204	0.012	6.0	86.6
24	13254738	69443056	0.191				

Appendix Table 27. Results of the incubation of ROS111 in human plasma over 24.00 h. Three samples were incubated over 24.00 h and measured at 265 nm with RP-UHPLC. The detected areas of ROS111 peaks were divided by the detected area of IS peaks (DOH). The average of the same time was converted to A(An)/A(IS). The calculated values were referred to the recovered ROS111 peak area at t = 0.00 h to receive the percentage of the analyte ROS111 (n = 3). IS – internal standard, DOH – Donepezil hydrochloride, Standard dev. – standard deviation, Var – coefficient of variation, A – peak area, An – analyte, RP-UHPLC - Reversed Phase High Performance Liquid Chromatography

	Peak area						
Time [h]	**ROS111**	**Donepezil**	**A(Compound)/A(Standard)**	**A/A mean**	**Standard dev.**	**Var. [%]**	**Degradation [%]**
0	312958	1367975	0.229				
0	315957	1349983	0.234	0.233	0.004	1.9	100.0
0	324956	1367527	0.238				
0.5	313046	1366534	0.229				
0.5	310427	1341628	0.231	0.230	0.001	0.5	98.6
0.5	314536	1366399	0.230				
1	314014	1364662	0.230				
1	310579	1364662	0.228	0.228	0.002	0.7	97.8
1	311633	1370288	0.227				
1.5	305503	1377534	0.222				
1.5	311962	1360218	0.229	0.227	0.004	1.9	97.2
1.5	308718	1345601	0.229				
2	306117	1365688	0.224				
2	307870	1367790	0.225	0.225	0.001	0.6	96.5
2	311302	1371759	0.227				
4	300931	1397831	0.215				
4	285180	1406804	0.203	0.210	0.006	3.0	89.8
4	296734	1406010	0.211				
24	263745	1377534	0.191				
24	268202	1360218	0.197	0.197	0.005	2.6	84.3
24	271476	1345601	0.202				

Appendix Table 28. Results of the incubation of ROS111 in rat plasma over 24.00 h. Three samples were incubated over 24.00 h and measured at 265 nm with RP-UHPLC. The detected areas of ROS111 peaks were divided by the detected area of IS peaks (DOH). The average of the same time was converted to A(An)/A(IS). The calculated values were referred to the recovered ROS111 peak area at t = 0.00 h to receive the percentage of the analyte ROS111 (n = 3). IS – internal standard, DOH – Donepezil hydrochloride, Standard dev. – standard deviation, Var – coefficient of variation, A – peak area, An – analyte, RP-UHPLC - Reversed Phase High Performance Liquid Chromatography

	Peak area						
Time [h]	**ROS111**	**Donepezil**	**A(Compound)/A(Standard)**	**A/A mean**	**Standard dev.**	**Var. [%]**	**Degradation [%]**
0	329051	1128326	0.292				
0	326073	1128130	0.289	0.296	0.010	3.5	100.0
0	345664	1121437	0.308				
0.5	313952	1103925	0.284				
0.5	335502	1103925	0.304	0.293	0.010	3.4	98.8
0.5	323977	1115893	0.290				
1	314714	1161082	0.271				
1	324102	1154363	0.281	0.276	0.005	1.8	93.1
1	318128	1154060	0.276				
1.5	312802	1146504	0.273				
1.5	321826	1130483	0.285	0.276	0.008	2.9	93.0
1.5	310934	1155042	0.269				
2	313509	1182639	0.265				
2	329218	1157937	0.284	0.275	0.010	3.5	92.9
2	315623	1141688	0.276				
4	307403	1134829	0.271				
4	307674	1112939	0.276	0.268	0.010	3.8	90.5
4	286623	1116418	0.257				
24	333524	1238339	0.269				
24	331122	1217363	0.272	0.266	0.009	3.4	89.6
24	336577	1318244	0.255				

Appendix Table 29. Results of the incubation of ROS114 in human plasma over 24.00 h. Three samples were incubated over 24.00 h and measured with the uV-detector of MS. The detected areas of ROS114 peaks were divided by the detected area of IS peaks (DOH). The average of the same time was converted to A(An)/A(IS). The calculated values were referred to the recovered ROS114 peak area at t = 0.00 h to receive the percentage of the analyte ROS114 (n = 3). MS – Mass Spectrometry, IS – internal standard, DOH – Donepezil hydrochloride, Standard dev. – standard deviation, Var – coefficient of variation, A – peak area, An – analyte

	Peak area						
Time [h]	ROS114	Donepezil	A(Compound)/A(Standard)	A/A mean	Standard dev.	Var. [%]	Degradation [%]
0	64056	114648	0.559				
0	63474	116790	0.543	0.562	0.020	3.6	100.0
0	66918	114699	0.583				
0.5	67909	118885	0.571				
0.5	64963	120565	0.539	0.554	0.016	2.9	98.6
0.5	64555	116919	0.552				
1	66622	120451	0.553				
1	66979	122724	0.546	0.551	0.004	0.8	98.0
1	67731	122375	0.553				
1.5	64063	121594	0.527				
1.5	67919	123256	0.551	0.542	0.013	2.4	96.4
1.5	66133	120943	0.547				
2	64473	119473	0.540				
2	65976	121927	0.541	0.538	0.005	0.8	95.7
2	64933	121924	0.533				
4	62112	118917	0.522				
4	63911	125084	0.511	0.518	0.006	1.2	92.2
4	63861	122413	0.522				
24	51191	117098	0.437				
24	55691	121130	0.460	0.450	0.012	2.6	80.1
24	58547	129396	0.452				

Appendix Table 30. Results of the incubation of ROS114 in rat plasma over 24.00 h. Three samples were incubated over 24.00 h and measured with MS. The detected areas of ROS114 peaks were divided by the detected area of IS peaks (DOH). The average of the same time was converted to A(An)/A(IS). The calculated values were referred to the recovered ROS114 peak area at t = 0.00 h to receive the percentage of the analyte ROS114 (n = 3). MS – Mass Spectrometry, IS – internal standard, DOH – Donepezil hydrochloride, Standard dev. – standard deviation, Var – coefficient of variation, A – peak area, An – analyte

	Peak area						
Time [h]	**ROS114**	**Donepezil**	**A(Compound)/A(Standard)**	**A/A mean**	**Standard dev.**	**Var. [%]**	**Degradation [%]**
0	10523214	58240116	0.181				
0	12208307	71313248	0.171	0.175	0.005	3.0	100.0
0	13735796	79953608	0.172				
0.5	12358045	72926744	0.169				
0.5	13931195	80870656	0.172	0.171	0.001	0.8	97.8
0.5	12916484	75734824	0.171				
1	11637740	69493352	0.167				
1	12368943	73936160	0.167	0.168	0.001	0.7	96.3
1	11053154	65261276	0.169				
1.5	10884793	67997744	0.160				
1.5	11812279	68987120	0.171	0.166	0.006	3.4	95.2
1.5	13282895	79436880	0.167				
2	14818151	86320296	0.172				
2	14785874	88919208	0.166	0.166	0.006	3.8	94.9
2	15248911	95823904	0.159				
4	11657563	73516496	0.159				
4	12476769	76052728	0.164	0.162	0.003	2.1	93.0
4	12874127	78199200	0.165				
24	11015492	83324848	0.132				
24	9719038	70213032	0.138	0.137	0.004	2.9	78.3
24	16814686	120427328	0.140				

Appendix Table 31. Results of the incubation of ROS150 in human plasma over 24.00 h. Three samples were incubated over 24.00 h and measured at 254 nm with RP-UHPLC. The detected areas of ROS150 peaks were divided by the detected area of IS peaks (DOH). The average of the same time was converted to A(An)/A(IS). The calculated values were referred to the recovered ROS150 peak area at t = 0.00 h to receive the percentage of the analyte ROS150 (n = 3). IS – internal standard, DOH – Donepezil hydrochloride, Standard dev. – standard deviation, Var – coefficient of variation, A – peak area, An – analyte, RP-UHPLC - Reversed Phase High Performance Liquid Chromatography

	Peak area						
Time [h]	**ROS150**	**Donepezil**	**A(Compound)/A(Standard)**	**A/A mean**	**Standard dev.**	**Var. [%]**	**Degradation [%]**
0	339727	781666	0.435				
0	368165	778672	0.473	0.450	0.020	4.5	100.0
0	342065	773781	0.442				
0.5	341096	772991	0.441				
0.5	349357	776338	0.450	0.442	0.007	1.6	98.3
0.5	336323	771779	0.436				
1	334553	774314	0.432				
1	346246	771331	0.449	0.432	0.016	3.8	96.1
1	323453	776903	0.416				
1.5	332177	784524	0.423				
1.5	339615	774276	0.439	0.432	0.008	1.8	95.9
1.5	335952	776596	0.433				
2	320193	773549	0.414				
2	338815	776669	0.436	0.429	0.013	3.0	95.4
2	339058	776476	0.437				
4	327261	784747	0.417				
4	329334	775997	0.424	0.415	0.011	2.6	92.2
4	314021	779382	0.403				
24	304759	790015	0.386				
24	294678	761208	0.387	0.397	0.018	4.6	88.3
24	317904	760030	0.418				

Appendix Table 32. Results of the incubation of ROS150 in rat plasma over 24.00 h. Three samples were incubated over 24.00 h and measured at 254 nm with RP-UHPLC. The detected areas of ROS150 peaks were divided by the detected area of IS peaks (DOH). The average of the same time was converted to A(An)/A(IS). The calculated values were referred to the recovered ROS150 peak area at t = 0.00 h to receive the percentage of the analyte ROS150 (n = 3). IS – internal standard, DOH – Donepezil hydrochloride, Standard dev. – standard deviation, Var – coefficient of variation, A – peak area, An – analyte, RP-UHPLC - Reversed Phase High Performance Liquid Chromatography

	Peak area						
Time [h]	**ROS150**	**Donepezil**	**A(Compound)/A(Standard)**	**A/A mean**	**Standard dev.**	**Var. [%]**	**Degradation [%]**
0	135401	345025	0.392				
0	131931	353089	0.374	0.388	0.012	3.2	100.0
0	136277	343135	0.397				
0.5	168254	459216	0.366				
0.5	151757	383790	0.395	0.382	0.015	3.8	98.4
0.5	138799	362345	0.383				
1	153251	425010	0.361				
1	179106	471176	0.380	0.373	0.011	3.0	96.3
1	175727	463365	0.379				
1.5	182017	479168	0.380				
1.5	167946	443790	0.378	0.372	0.012	3.1	96.1
1.5	192964	537368	0.359				
2	175876	493301	0.357				
2	208884	536859	0.389	0.372	0.016	4.4	95.9
2	197599	534029	0.370				
4	196671	553272	0.355				
4	223117	612692	0.364	0.364	0.008	2.3	93.9
4	229300	616168	0.372				
24	209123	730334	0.286				
24	218562	719985	0.304	0.296	0.009	3.0	76.4
24	196446	657126	0.299				

Appendix Table 33. Results of the incubation of ROS151 in human plasma over 24.00 h. Three samples were incubated over 24.00 h and measured at 300 nm with RP-UHPLC. The detected areas of ROS151 peaks were divided by the detected area of IS peaks (DOH). The average of the same time was converted to A(An)/A(IS). The calculated values were referred to the recovered ROS151 peak area at t = 0.00 h to receive the percentage of the analyte ROS151 (n = 3). IS – internal standard, DOH – Donepezil hydrochloride, Standard dev. – standard deviation, Var – coefficient of variation, A – peak area, An – analyte, RP-UHPLC - Reversed Phase High Performance Liquid Chromatography

	Peak area						
Time [h]	**ROS151**	**Donepezil**	**A(Compound)/A(Standard)**	**A/A mean**	**Standard dev.**	**Var. [%]**	**Degradation [%]**
0	223457	986000	0.227				
0	225104	990334	0.227	0.228	0.002	0.7	100.0
0	224302	977167	0.230				
0.5	224885	985839	0.228				
0.5	220171	975156	0.226	0.225	0.004	1.6	98.8
0.5	222166	1004274	0.221				
1	218457	993704	0.220				
1	222022	994786	0.223	0.222	0.002	0.9	97.5
1	222099	995235	0.223				
1.5	218296	1004131	0.217				
1.5	219835	979418	0.224	0.221	0.004	1.6	97.0
1.5	219281	991989	0.221				
2	218307	1004131	0.217				
2	220214	979418	0.225	0.218	0.006	2.7	95.9
2	211264	991989	0.213				
4	208069	978700	0.213				
4	208272	984760	0.211	0.212	0.001	0.3	93.0
4	223903	1059053	0.211				
24	196862	986000	0.200				
24	202488	990334	0.204	0.203	0.003	1.4	89.1
24	200097	977167	0.205				

Appendix Table 34. Results of the incubation of ROS151 in rat plasma over 24.00 h. Three samples were incubated over 24.00 h and measured at 300 nm with RP-UHPLC. The detected areas of ROS151 peaks were divided by the detected area of IS peaks (DOH). The average of the same time was converted to A(An)/A(IS). The calculated values were referred to the recovered ROS151 peak area at t = 0.00 h to receive the percentage of the analyte ROS151 (n = 3). IS – internal standard, DOH – Donepezil hydrochloride, Standard dev. – standard deviation, Var – coefficient of variation, A – peak area, An – analyte, RP-UHPLC - Reversed Phase High Performance Liquid Chromatography

	Peak area						
Time [h]	**ROS151**	**Donepezil**	**A(Compound)/A(Standard)**	**A/A mean**	**Standard dev.**	**Var. [%]**	**Degradation [%]**
0	233525	721398	0.324				
0	234664	738209	0.318	0.320	0.004	1.1	100.0
0	234664	739698	0.317				
0.5	222709	727043	0.306				
0.5	230995	739329	0.312	0.312	0.006	1.9	97.7
0.5	232625	730380	0.318				
1	225268	725170	0.311				
1	223885	734352	0.305	0.310	0.005	1.6	97.1
1	225730	716455	0.315				
1.5	224037	737696	0.304				
1.5	222644	735801	0.303	0.306	0.005	1.5	95.7
1.5	228741	735457	0.311				
2	219472	737129	0.298				
2	219130	733989	0.299	0.296	0.004	1.3	92.6
2	218857	750209	0.292				
4	217969	749384	0.291				
4	218197	752994	0.290	0.292	0.004	1.3	91.5
4	217066	731460	0.297				
24	150136	725093	0.207				
24	140283	741204	0.189	0.206	0.016	7.9	64.4
24	161719	729963	0.222				

Appendix Table 35. Results of the incubation of SON38 in human plasma over 24.00 h. Three samples were incubated over 24.00 h and measured at 260 nm with RP-UHPLC. The detected areas of SON38 peaks were divided by the detected area of IS peaks (DOH). The average of the same time was converted to A(An)/A(IS). The calculated values were referred to the recovered SON38 peak area at t = 0.00 h to receive the percentage of the analyte SON38 (n = 3). IS – internal standard, DOH – Donepezil hydrochloride, Standard dev. – standard deviation, Var – coefficient of variation, A – peak area, An – analyte, RP-UHPLC - Reversed Phase High Performance Liquid Chromatography

	Peak area						
Time [h]	**SON38**	**Donepezil**	**A(Compound)/A(Standard)**	**A/A mean**	**Standard dev.**	**Var. [%]**	**Degradation [%]**
0	250360	1121804	0.223				
0	270958	1138422	0.238	0.232	0.008	3.5	100.0
0	262809	1112910	0.236				
0.5	246462	1135980	0.217				
0.5	247707	1138114	0.218	0.223	0.010	4.3	95.8
0.5	263676	1127944	0.234				
1	240486	1125961	0.214				
1	253108	1150129	0.220	0.219	0.004	2.0	94.0
1	240486	1082520	0.222				
1.5	245771	1140131	0.216				
1.5	247551	1131570	0.219	0.218	0.002	0.8	93.6
1.5	247054	1131853	0.218				
2	240117	1105492	0.217				
2	240960	1100105	0.219	0.216	0.004	1.8	92.9
2	243062	1148639	0.212				
4	247146	1138102	0.217				
4	245599	1132873	0.217	0.216	0.002	0.9	92.8
4	247363	1158864	0.213				
24	248358	1138102	0.218				
24	237940	1132873	0.210	0.212	0.005	2.4	91.3
24	241821	1158864	0.209				

Appendix Table 36. Results of the incubation of SON38 in rat plasma over 24.00 h. Three samples were incubated over 24.00 h and measured with MS. The detected areas of SON38 peaks were divided by the detected area of IS peaks (DOH). The average of the same time was converted to A(An)/A(IS). The calculated values were referred to the recovered SON38 peak area at t = 0.00 h to receive the percentage of the analyte SON38 (n = 3). MS – Mass Spectrometry, IS – internal standard, DOH – Donepezil hydrochloride, Standard dev. – standard deviation, Var – coefficient of variation, A – peak area, An – analyte

	Peak area						
Time [h]	**SON38**	**Donepezil**	**A(Compound)/A(Standard)**	**A/A mean**	**Standard dev.**	**Var. [%]**	**Degradation [%]**
0	61611008	93908928	0.656				
0	24264314	36951288	0.657	0.652	0.008	1.2	100.0
0	25457950	39636584	0.642				
0.5	30031410	45866256	0.655				
0.5	35441452	53942464	0.657	0.644	0.020	3.2	98.8
0.5	32317938	52078796	0.621				
1	46463908	73600056	0.631				
1	28974018	47100284	0.615	0.631	0.016	2.5	96.8
1	14182182	21944126	0.646				
1.5	20979350	33013592	0.635				
1.5	14736256	24572370	0.600	0.620	0.019	3.0	95.2
1.5	17651218	28191784	0.626				
2	36415292	59699524	0.610				
2	69601984	121846160	0.571	0.590	0.019	3.3	90.6
2	36832736	62453488	0.590				
4	36027688	60522304	0.595				
4	53352132	97402960	0.548	0.577	0.026	4.4	88.5
4	30231722	51409860	0.588				
24	13373340	27558870	0.485				
24	43162060	89642536	0.481	0.471	0.022	4.7	72.2
24	14284867	32110100	0.445				

Appendix Table 37. Results of the incubation of SON39 in human plasma over 24.00 h. Three samples were incubated over 24.00 h and measured with MS. The detected areas of SON39 peaks were divided by the detected area of IS peaks (DOH). The average of the same time was converted to A(An)/A(IS). The calculated values were referred to the recovered SON39 peak area at t = 0.00 h to receive the percentage of the analyte SON39 (n = 3). MS – Mass Spectrometry, IS – internal standard, DOH – Donepezil hydrochloride, Standard dev. – standard deviation, Var – coefficient of variation, A – peak area, An – analyte

	Peak area						
Time [h]	**SON39**	**Donepezil**	**A(Compound)/A(Standard)**	**A/A mean**	**Standard dev.**	**Var. [%]**	**Degradation [%]**
0	69420528	86643544	0.801				
0	45333308	57176280	0.793	0.792	0.010	1.2	100.0
0	44100904	56403132	0.782				
0.5	57344692	76646144	0.748				
0.5	36769260	47182420	0.779	0.762	0.016	2.1	96.2
0.5	48461072	63980064	0.757				
1	58890892	81923616	0.719				
1	18804004	24350272	0.772	0.747	0.027	3.6	94.3
1	16450568	21979068	0.748				
1.5	41878100	58874172	0.711				
1.5	20197728	26751184	0.755	0.740	0.025	3.3	93.4
1.5	11717529	15564084	0.753				
2	53475644	74298936	0.720				
2	56473836	75137832	0.752	0.735	0.016	2.2	92.8
2	52120748	71117504	0.733				
4	45717056	64177424	0.712				
4	54008868	73273288	0.737	0.722	0.013	1.9	91.1
4	52914412	73908680	0.716				
24	50345472	69995952	0.719				
24	53410456	75888008	0.704	0.716	0.011	1.5	90.4
24	49964524	68943488	0.725				

Appendix Table 38. Results of the incubation of SON39 in rat plasma over 24.00 h. Three samples were incubated over 24.00 h and measured with MS. The detected areas of SON39 peaks were divided by the detected area of IS peaks (DOH). The average of the same time was converted to A(An)/A(IS). The calculated values were referred to the recovered SON39 peak area at t = 0.00 h to receive the percentage of the analyte SON39 (n = 3). MS – Mass Spectrometry, IS – internal standard, DOH – Donepezil hydrochloride, Standard dev. – standard deviation, Var – coefficient of variation, A – peak area, An – analyte

	Peak area						
Time [h]	**SON39**	**Donepezil**	**A(Compound)/A(Standard)**	**A/A mean**	**Standard dev.**	**Var. [%]**	**Degradation [%]**
0	39651820	67517144	0.587				
0	39183756	69576904	0.563	0.579	0.014	2.4	100.0
0	40241988	68586600	0.587				
0.5	49010632	85600000	0.573				
0.5	44355732	76875536	0.577	0.578	0.005	0.9	99.8
0.5	42142260	72234544	0.583				
1	40811884	73219376	0.557				
1	45528468	79221944	0.575	0.572	0.013	2.2	98.7
1	38627556	66319572	0.582				
1.5	40031464	73453584	0.545				
1.5	40371148	72806112	0.555	0.560	0.018	3.3	96.7
1.5	40016928	68953760	0.580				
2	43391428	77680176	0.559				
2	43076252	76341424	0.564	0.555	0.012	2.2	95.8
2	42086192	77780648	0.541				
4	39122816	76795584	0.509				
4	44178416	80457848	0.549	0.522	0.024	4.5	90.1
4	31222550	61608668	0.507				
24	80118008	163006320	0.492				
24	104059976	197506336	0.527	0.519	0.024	4.7	89.6
24	107156208	199077488	0.538				

Appendix Table 39. Results of the incubation of VA2 in human plasma over 24.00 h. Three samples were incubated over 24.00 h and measured at 254 nm with RP-UHPLC. The detected areas of VA2 peaks were divided by the detected area of IS peaks (DOH). The average of the same time was converted to A(An)/A(IS). The calculated values were referred to the recovered VA2 peak area at t = 0.00 h to receive the percentage of the analyte VA2 (n = 3). IS – internal standard, DOH – Donepezil hydrochloride, Standard dev. – standard deviation, Var – coefficient of variation, A – peak area, An – analyte, RP-UHPLC - Reversed Phase High Performance Liquid Chromatography

	Peak area						
Time [h]	**VA2**	**Donepezil**	**A(Compound)/A(Standard)**	**A/A mean**	**Standard dev.**	**Var. [%]**	**Degradation [%]**
0	290838	789319	0.368				
0	293901	793525	0.370	0.373	0.006	1.6	100.0
0	297418	783088	0.380				
0.5	293699	787702	0.373				
0.5	293367	777299	0.377	0.370	0.008	2.3	99.3
0.5	280946	778084	0.361				
1	286010	767834	0.372				
1	280511	767416	0.366	0.370	0.004	1.0	99.1
1	286362	772768	0.371				
1.5	286602	777121	0.369				
1.5	286982	777949	0.369	0.366	0.006	1.6	98.0
1.5	283237	789108	0.359				
2	278393	789443	0.353				
2	280388	777049	0.361	0.362	0.010	2.9	97.2
2	288080	771608	0.373				
4	272502	784428	0.347				
4	269808	764774	0.353	0.352	0.004	1.2	94.4
4	272497	766888	0.355				
24	279068	807362	0.346				
24	267603	779944	0.343	0.343	0.002	0.6	92.1
24	263418	771456	0.341				

Appendix Table 40. Results of the incubation of VA2 in rat plasma over 24.00 h. Three samples were incubated over 24.00 h and measured at 254 nm with RP-UHPLC. The detected areas of VA2 peaks were divided by the detected area of IS peaks (DOH). The average of the same time was converted to A(An)/A(IS). The calculated values were referred to the recovered VA2 peak area at t = 0.00 h to receive the percentage of the analyte VA2 (n = 3). IS – internal standard, DOH – Donepezil hydrochloride, Standard dev. – standard deviation, Var – coefficient of variation, A – peak area, An – analyte, RP-UHPLC - Reversed Phase High Performance Liquid Chromatography

	Peak area						
Time [h]	**VA2**	**Donepezil**	**A(Compound)/A(Standard)**	**A/A mean**	**Standard dev.**	**Var. [%]**	**Degradation [%]**
0	267855	600583	0.446				
0	272191	598955	0.454	0.452	0.005	1.1	100.0
0	272402	598803	0.455				
0.5	268188	603080	0.445				
0.5	266030	595366	0.447	0.444	0.003	0.6	98.4
0.5	264363	598590	0.442				
1	263518	602782	0.437				
1	264692	602587	0.439	0.438	0.001	0.2	97.0
1	263862	601469	0.439				
1.5	254422	596783	0.426				
1.5	261996	591790	0.443	0.431	0.010	2.4	95.4
1.5	256594	605587	0.424				
2	253998	611074	0.416				
2	255974	601304	0.426	0.420	0.005	1.2	93.0
2	254024	605161	0.420				
4	250274	607875	0.412				
4	252794	607875	0.416	0.414	0.002	0.5	91.7
4	250684	603631	0.415				
24	243036	605003	0.402				
24	242608	606421	0.400	0.401	0.001	0.2	88.8
24	243118	606100	0.401				

Appendix Table 41. Results of the incubation of VIT10 in human plasma over 24.00 h. Three samples were incubated over 24.00 h and measured with MS. The detected areas of VIT10 peaks were divided by the detected area of IS peaks (DOH). The average of the same time was converted to A(An)/A(IS). The calculated values were referred to the recovered VIT10 peak area at t = 0.00 h to receive the percentage of the analyte VIT10 (n = 3). MS – Mass Spectrometry, IS – internal standard, DOH – Donepezil hydrochloride, Standard dev. – standard deviation, Var – coefficient of variation, A – peak area, An – analyte

	Peak area						
Time [h]	**VIT10**	**Donepezil**	**A(Compound)/A(Standard)**	**A/A mean**	**Standard dev.**	**Var. [%]**	**Degradation [%]**
0	25397604	77597200	0.327				
0	20151388	60394884	0.334	0.331	0.003	1.0	100.0
0	26708072	80350216	0.332				
0.5	24117914	74489392	0.324				
0.5	22049562	67697080	0.326	0.330	0.009	2.7	99.6
0.5	23994210	70544136	0.340				
1	29809796	90861080	0.328				
1	24251438	74419048	0.326	0.328	0.002	0.5	98.9
1	30670772	93282048	0.329				
1.5	21739032	71614000	0.304				
1.5	25550050	81179024	0.315	0.312	0.008	2.6	94.4
1.5	26263380	82320408	0.319				
2	23660824	75380264	0.314				
2	24156986	80882616	0.299	0.308	0.008	2.7	93.1
2	26243662	84083976	0.312				
4	25960590	83532264	0.311				
4	25240776	83074136	0.304	0.307	0.003	1.1	92.8
4	24949104	81217352	0.307				
24	22050454	77221784	0.286				
24	22694012	83627720	0.271	0.277	0.008	2.7	83.7
24	20903806	76232792	0.274				

Appendix Table 42. Results of the incubation of VIT10 in rat plasma over 24.00 h. Three samples were incubated over 24.00 h and measured with MS. The detected areas of VIT10 peaks were divided by the detected area of IS peaks (DOH). The average of the same time was converted to A(An)/A(IS). The calculated values were referred to the recovered VIT10 peak area at t = 0.00 h to receive the percentage of the analyte VIT10 (n = 3). MS – Mass Spectrometry, IS – internal standard, DOH – Donepezil hydrochloride, Standard dev. – standard deviation, Var – coefficient of variation, A – peak area, An – analyte

	Peak area						
Time [h]	**VIT10**	**Donepezil**	**A(Compound)/A(Standard)**	**A/A mean**	**Standard dev.**	**Var. [%]**	**Degradation [%]**
0	15933347	63369504	0.251				
0	20465000	79778216	0.257	0.260	0.010	4.0	100.0
0	17951334	66147548	0.271				
0.5	16788468	66766392	0.251				
0.5	22395562	86716936	0.258	0.257	0.005	2.1	99.0
0.5	18367944	70140936	0.262				
1	17214158	67300616	0.256				
1	16113917	65030936	0.248	0.256	0.009	3.4	98.7
1	18535124	69832592	0.265				
1.5	15584572	64792704	0.241				
1.5	15833989	65763228	0.241	0.248	0.012	4.9	95.3
1.5	21304972	81458120	0.262				
2	18868844	78224096	0.241				
2	19870276	79486104	0.250	0.244	0.005	2.2	93.9
2	10473697	43591004	0.240				
4	16407098	77815120	0.211				
4	17474898	78510912	0.223	0.219	0.007	3.4	84.5
4	18566714	82602528	0.225				
24	21303306	204852448	0.104				
24	22947118	212647568	0.108	0.106	0.002	2.0	41.0
24	22121534	206284416	0.107				

Appendix Table 43. Results of the incubation of VIT34A in human plasma over 24.00 h. Three samples were incubated over 24.00 h and measured with MS. The detected areas of VIT34A peaks were divided by the detected area of IS peaks (DOH). The average of the same time was converted to A(An)/A(IS). The calculated values were referred to the recovered VIT34A peak area at t = 0.00 h to receive the percentage of the analyte VIT34A (n = 3). MS – Mass Spectrometry, IS – internal standard, DOH – Donepezil hydrochloride, Standard dev. – standard deviation, Var – coefficient of variation, A – peak area, An – analyte

	Peak area						
Time [h]	VIT34A	Donepezil	A(Compound)/A(Standard)	A/A mean	Standard dev.	Var. [%]	Degradation [%]
0	87404	116098	0.753				
0	86954	115515	0.753	0.748	0.008	1.0	100.0
0	84510	114246	0.740				
0.5	86502	122269	0.707				
0.5	85630	121815	0.703	0.716	0.018	2.5	95.6
0.5	90593	123039	0.736				
1	83644	119165	0.702				
1	88183	123640	0.713	0.714	0.012	1.7	95.3
1	87274	120259	0.726				
1.5	88205	123708	0.713				
1.5	87736	128595	0.682	0.706	0.022	3.1	94.4
1.5	87840	121315	0.724				
2	76782	111521	0.688				
2	76481	113755	0.672	0.667	0.025	3.7	89.1
2	78314	122465	0.639				
4	81287	124115	0.655				
4	81971	124699	0.657	0.659	0.005	0.8	88.0
4	77376	116430	0.665				
24	74930	121274	0.618				
24	73585	118336	0.622	0.624	0.007	1.1	83.3
24	80713	127843	0.631				

Appendix Table 44. Results of the incubation of VIT34A in rat plasma over 24.00 h. Three samples were incubated over 24.00 h and measured with MS. The detected areas of VIT34A peaks were divided by the detected area of IS peaks (DOH). The average of the same time was converted to A(An)/A(IS). The calculated values were referred to the recovered VIT34A peak area at t = 0.00 h to receive the percentage of the analyte VIT34A (n = 3). MS – Mass Spectrometry, IS – internal standard, DOH – Donepezil hydrochloride, Standard dev. – standard deviation, Var – coefficient of variation, A – peak area, An – analyte

	Peak area						
Time [h]	**VIT34A**	**Donepezil**	**A(Compound)/A(Standard)**	**A/A mean**	**Standard dev.**	**Var. [%]**	**Degradation [%]**
0	13180268	79537056	0.166				
0	13793858	77199080	0.179	0.172	0.006	3.8	100.0
0	13263236	77329048	0.172				
0.5	12292884	76197064	0.161				
0.5	14929334	84015392	0.178	0.170	0.008	4.8	98.7
0.5	13866409	81540448	0.170				
1	11866979	73936256	0.161				
1	11765297	67660312	0.174	0.169	0.007	4.3	98.2
1	12823100	74440800	0.172				
1.5	11013102	67982400	0.162				
1.5	13613660	77786656	0.175	0.168	0.007	4.0	97.5
1.5	12662964	76192264	0.166				
2	12359585	78156592	0.158				
2	11259950	67406904	0.167	0.163	0.004	2.7	94.5
2	12694268	78193848	0.162				
4	13601091	86038576	0.158				
4	13769824	86345424	0.159	0.161	0.004	2.7	93.8
4	12976723	78035600	0.166				
24	20033838	215019728	0.093				
24	21070652	207664368	0.101	0.098	0.004	4.3	56.7
24	21999278	224367072	0.098				

Appendix Table 45. Results of the incubation of WIN13 in human plasma over 24.00 h. Three samples were incubated over 24.00 h and measured with MS. The detected areas of WIN13 peaks were divided by the detected area of IS peaks (DOH). The average of the same time was converted to A(An)/A(IS). The calculated values were referred to the recovered WIN13 peak area at t = 0.00 h to receive the percentage of the analyte WIN13 (n = 3). MS – Mass Spectrometry, IS – internal standard, DOH – Donepezil hydrochloride, Standard dev. – standard deviation, Var – coefficient of variation, A – peak area, An – analyte

	Peak area						
Time [h]	WIN13	Donepezil	A(Compound)/A(Standard)	A/A mean	Standard dev.	Var. [%]	Degradation [%]
0	10451876	18397430	0.568				
0	41716248	70040288	0.596	0.584	0.014	2.4	100.0
0	46549484	79157864	0.588				
0.5	32867058	59047076	0.557				
0.5	18681610	31640644	0.590	0.577	0.018	3.1	98.8
0.5	48185512	82429976	0.585				
1	37758068	68550048	0.551				
1	22165822	37618532	0.589	0.572	0.019	3.4	97.9
1	43123956	75054688	0.575				
1.5	41979796	76037072	0.552				
1.5	42987220	72525248	0.593	0.570	0.021	3.6	97.7
1.5	48564328	85800112	0.566				
2	36203656	65795124	0.550				
2	38246084	67142512	0.570	0.557	0.011	2.0	95.3
2	44656788	81150224	0.550				
4	37256112	69659184	0.535				
4	43847544	79501272	0.552	0.547	0.010	1.9	93.6
4	38871888	70217528	0.554				
24	46189684	85047696	0.543				
24	45220524	82579032	0.548	0.539	0.012	2.1	92.3
24	44531940	84701008	0.526				

Appendix Table 46. Results of the incubation of WIN13 in rat plasma over 24.00 h. Three samples were incubated over 24.00 h and measured with MS. The detected areas of WIN13 peaks were divided by the detected area of IS peaks (DOH). The average of the same time was converted to A(An)/A(IS). The calculated values were referred to the recovered WIN13 peak area at t = 0.00 h to receive the percentage of the analyte WIN13 (n = 3). MS – Mass Spectrometry, IS – internal standard, DOH – Donepezil hydrochloride, Standard dev. – standard deviation, Var – coefficient of variation, A – peak area, An – analyte

	Peak area						
Time [h]	WIN13	Donepezil	A(Compound)/A(Standard)	A/A mean	Standard dev.	Var. [%]	Degradation [%]
0	38846856	72536272	0.536				
0	36105024	69655096	0.518	0.523	0.011	2.1	100.0
0	34675392	67350264	0.515				
0.5	38219740	76490040	0.500				
0.5	31378086	60020860	0.523	0.505	0.016	3.1	96.6
0.5	36692176	74405168	0.493				
1	30615608	63146912	0.485				
1	40152664	77809608	0.516	0.499	0.016	3.2	95.5
1	32905284	66234256	0.497				
1.5	47806712	99876592	0.479				
1.5	48581392	97913456	0.496	0.495	0.016	3.3	94.7
1.5	44492980	86988624	0.511				
2	48690940	98467584	0.494				
2	42461764	85975080	0.494	0.494	0.000	0.1	94.5
2	41809048	84629808	0.494				
4	41606952	89323840	0.466				
4	38186984	76577368	0.499	0.481	0.017	3.4	92.0
4	40619612	84872600	0.479				
24	33222588	74760560	0.444				
24	36835112	78846800	0.467	0.466	0.021	4.5	89.1
24	36353276	74730904	0.486				

Appendix Table 47. The percentages of the analytes were estimated by relating the peak areas of the analytes against the respective IS. For the analysis, the calculated ratio of the analytes at t = 0.00 h was assumed to be 100%. The metabolic stability was assessed by plotting the incubation time (x-axis) against the percentage of remaining analyte (y-axis). In order to calculate the in vitro $t^1/_2$ the incubation time was plotted against the natural logarithm of the percentage of the analyte remaining in the plasma. The linear part of the slope was used for the in vitro $t^1/_2$ by calculating ln2/k. $t^1/_2$ - half-life, k – elimination rate constant

analyte	human plasma $t^1/_2$ [h]	rat plasma $t^1/_2$ [h]
AN13	173	59
AN15	161	51
GIA5	248	110
GIA13	277	154
GIA14	866	88
GIA17	433	385
GIA22	187	74
GIA25	144	187
MCC9	330	42
MCC13	78	58
MCC17	462	47
MCC20	77	43
MCS2	193	131
ROS111	107	239
ROS114	80	75
ROS131	39	17
ROS150	165	65
ROS151	169	39
SON38	347	55
SON39	289	173
VA2	231	182
VIT8	47	18
VIT10	105	18
VIT34A	120	29
VIT35	55	62
WIN13	267	204